Surgical stress attenuates pre-existing anti-tumour immunity resulting in postoperative metastases and local recurrence in a murine model

By

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ABSTRACT

Solid malignancies in cancer patients require surgical intervention; however, surgery has been shown to promote the metastatic potential of tumour cells. Surgery-induced impairment of adaptive immunity is poorly understood, thus, our aim is to characterize the impact of surgery on tumour antigen-specific cytotoxic T lymphocyte function. To generate anti-tumour immunity, we adopted a C57/B6 model of B16 melanoma immunized with intramuscular (IM) AdhDCT, an adenovirus expressing the melanoma-associated antigen human dopachrome tautomerase (hDCT). Surgical stress was induced by left abdominal nephrectomy. We found that surgery reduces overall survival in AdhDCT-immunized mice, whereas those that did not undergo surgery were cured of their tumours. Surgical stress also decreases both the proportion and absolute spleen numbers of DCT-specific IFN-gamma+ CD8+ T-cells by over 2-fold. We have shown that perioperative suppression of antigen-specific T-cells can lead to increased tumour burden in a murine melanoma model.

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LIST OF ABBREVIATIONS

ACT = Adoptive cell transfer

APC = Antigen presenting cell

CD = Cluster of differentiation

CTLA4 = Cytotoxic T-lymphocyte antigen 4

DAMP = Damage associated molecular pattern

DC = Dendritic cell

DCT = dopachrome tautomerase

GM-CSF = Grannulocyte-monocyte colony stimulating factor

HLA = Human leukocyte antigen

IFN = Interferon

IL = Interleukin

IP = Intraperitoneal

IV = Intravenous

MDSC = Meyloid-derived suppressor cells

MHC = Major histocompatibility complex

MRD – Minimal residual disease

NK = Natural killer

PAMP = pathogen associate molecular pattern

PBS = Phosphate-buffered saline

PFU = Plaque forming units

PRR = Pattern recognition receptors

TAA = Tumour associated antigen

TAP = Transporters associated with antigen processing

TDLN = Tumour-draining lymph node

TGFβ = Transforming growth factor β

TIL = Tumour infiltrating lymphocyte

TLR = Toll-like receptor

TNF = Tumour necrosis family

Treg = Regulatory T-cell

VSV = Vesicular stomatitis virus

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1 - INTRODUCTION

1.1 Cancer

In Canada, cancer is the leading cause of death, responsible for nearly 30% of all deaths, followed by cardiovascular diseases and chronic lower respiratory diseases[1]. Almost half of all Canadians will develop cancer in their lifetime and about a quarter of all Canadians will succumb to this disease[1].

Cancer is a complex disease that can arise in several ways, including exposure to carcinogens, radiation, viral infections, chronic inflammation, and heritable mutations. However, fundamentally, cancer is a genetic disease; mutations in the genome that lead to abnormalities in the cell cycle can initiate the aberrant proliferation of cells with the potential of invading other tissues. Over the years, the definition of cancer has evolved, and currently, the disease can be characterized by ten hallmarks: (1) cancer cells stimulate their own growth; (2) they resist inhibitory signals that might prohibit their growth; (3) they resist apoptosis; (4) they promote angiogenesis; (5) they divide perpetually; (6) they invade local tissues and metastasize to distant sites; (7) they have abnormal metabolic pathways; (8) they have unstable genomes and chromosome aberrancies; (9) they promote inflammation; and (10) cancer cells evade the immune system[2,3].

1.2 The Immune System

The immune system plays an essential role in the defence against cancer, therefore the ability of cancer cells to evade the immune system has been of major research focus in

the last decade. The immune system consists of an organization of cells and molecules which functions to protect us against foreign pathogens or abnormal entities, including bacteria, parasites, viruses, and transformed cells[4]. The immune system's ability to distinguish between "self" and "non-self" is central to the immune response.

Broadly, the immune system is divided into two branches: the innate immune response and the adaptive immune response. The innate immune system is the first line of defence against pathogens and is both non-specific and requires no prior sensitization. Some of the cells of the innate immune system include dendritic cells (DCs), natural killer (NK) cells, and macrophages. In contrast, adaptive immunity is acquired through an initial interaction with an antigen, resulting in enduring immunologic memory and a specific response upon subsequent encounters with that same antigen. Cells of the adaptive immune system are primarily B-cells and T-cells[4].

The proper function of the immune system is of crucial importance in protecting the host from aberrant T-cells. In response, cancer cells have evolved complex mechanisms to evade the immune system or actively suppress it.

1.2.1 Cancer Immune Surveillance

Though the immune system was previously thought to be a driving force in tumorigenesis through the workings of chronic inflammation[5], it is now also seen as a source of natural anti-tumour defences that must be overcome or suppressed during neoplastic transformation[6,7]. This reciprocal interplay between the tumour and the immune

system is known as the process of cancer immunoediting, and has been divided into three distinct phases known as the three Es: elimination, equilibrium, and escape[8].

The initial step of elimination describes the process of immune surveillance, whereby the immune system can recognize and subsequently suppress or kill nascent tumours[8]. Recognition of cancer cells can be through the release of endogenous damage-associated molecular patterns (DAMPs)[9], the increase in activating NK cell ligands or the decrease in inhibitory NK ligands[10], and the expression of altered self-proteins that can be recognized by cells of the adaptive immune system[11].

DCs and other antigen presenting cells (APCs) generally initiate the process of immune surveillance through the phagocytosis of dying tumour cells. Danger signals such as DAMPs or pathogen-associated molecular patterns (PAMPs) bind to pattern recognition receptors (PRRs) on DCs and trigger their activation and maturation[12]. These activation signals trigger DCs to upregulate the expression of co-stimulatory molecules such as CD40, CD80, and CD86 , as well as secrete pro-inflammatory cytokines such as IL-12 and interferons (IFN)[12]. During maturation, DCs traffic to the draining lymph node where they digest tumour proteins and present them as peptides on MHC class I or class II along with co-stimulatory molecules. At this point, the DC interacts with CD4+ helper T-cells, which release important cytokines and survival signals. These licence the APC to continue driving the pro-inflammatory process and activate CD8+ effector T-cells, which then secrete cytokines such as TNFα and IFNγ and kill tumour cells in an antigen-dependent manner[13]. DCs can also initiate NK cell responses through the release of IL-2, IL-12, IL-15, and type I IFNs[9].

Different immune cell populations have evolved various mechanisms to recognize and kill tumour cells. NK cells recognize the increase in activating ligands, such as NKG2D, which can be induced through bacterial or viral infection or through cellular transformation[9]. They also recognize the loss of inhibitory ligands, like MHC class I, which is the basis of the "missing self" hypothesis[9]. B-cells can produce tumour-specific antibodies that lead to cancer cell death by blocking importanT-cellular receptors or initiating the complement cascade. Activated CD8+ T-cells recognize tumour cells based on the expression of tumour associated antigens (TAAs) that are presented via MHC class I. Though NK cells and CD8+ T-cells recognize tumours in different ways, they both kill tumour cells using similar mechanisms. Both release perforin which forms a pore in the targeT-cell and allows the entry of Granzyme B, a proteolytic enzyme that initiates apoptosis[6]. Also, both NK and T-cells produce an array of cytokines that have immune modulating and direct anti-cancer functions. Interferon gamma (IFNγ), the only type II IFN, is a very important cytokine with potent anti-tumour effects and is widely produced by activated NK and T-cells, among others. This cytokine targets tumour cells as well as the tumour's stromal counterparts, such as endothelial cells and immune cells. Through direct interaction with tumour cells, IFNγ has been demonstrated to increase MHC class I expression[14,15], thereby augmenting tumour cell immunogenicity. In addition, IFNγ recruits DCs, macrophages, NK cells, and T-cells to the tumour site.

Type I IFNs (IFNα and IFNβ especially) also play an important role in cancer rejection and are produced by most T-cells following binding of PRRs to viral or bacterial components. Type I IFNs can lead to increased DC phagocytosis, maturation, and antigen

presentation, in addition to increasing B-cell maturation and antibody production. These cytokines also enhance NK cell effector functions. In addition, type I IFNs can upregulate MHC class I expression, making tumours more visible to T-cells[16].

However, despite the immune system's best defences, some tumour cell variants adapt to the selective immune pressures and enter the second phase of cancer immunoediting, the equilibrium phase. Often said to be the longest-lasting, this phase is characterized by a balance between tumour growth and immune-mediated killing and can correlate with an apparent dormancy in the tumour[7].

In the escape phase, the tumour cell variants that escaped immune recognition begin to expand in an uncontrolled manner. Commonly, tumour cells downregulate or lose expression of MHC class I molecules in order to elude T-cell mediated killing[17]. Tumour cells can also develop defects in molecules necessary for antigen processing and presentation such as TAP protein, β2-microglobulin protein, and LMP2[18]. In addition, tumours cells also employ mechanisms to resist CTL-mediated killing by downregulation of death receptors and mutation of genes encoding apoptosis-inducing proteins such as caspase-8[19]. These are several among many strategies that are employed by tumour cells to evade immune recognition.

Tumour cell products also actively suppress the immune response. In an experimental mouse model, tumour-specific $CD8^+$ T-cells were shown to be active at the initial stages of tumour development, but typically showed loss of cytolytic function as the tumour developed[20]. Similarly, $CD4^+$ T-cells have also been shown to progressively lose their

anti-tumour activity[21]. Numerous factors secreted by tumour beds act to inhibit the maturation or function of various immune cells. Some of these factors include VEGF, interleukin-6 (IL-6), interleukin-10 (IL-10), and TGF-β[22]. TGF-β has numerous roles in suppressing the immune system such as inhibiting antigen presentation, T-cell proliferation, inhibiting NK cell cytotoxicity, and activating regulatory T (Treg) cells[8]. Treg cells are found in great numbers in the tumour microenvironment and act to suppress activation of CTLs and the immunosuppressive cytokine IL-10 can reduce DC development and activity[8]. The mechanisms described above demonstrate the ability of cancer cells to impede the generation of an adaptive immune response at many stages.

Another property of tumour cells is the ability to induce T-cell tolerance. If tumour cells do not provide the required activation signal for DC maturation, DCs are unable to express sufficient levels of co-stimulatory molecules required for T-cell activation leading to T-cell tolerance. As such, these tolerized T-cells are unable to mount a response against the target[23].

Altogether, these studies show some of the numerous ways that tumour cells work to evade the immune system. Less immunogenic tumour cell variants are selected for their ability to evade immune cell killing. In addition, active suppression of the immune system occurs by tumour cell secretions. Finally, cancer cells are able to induce T-cell tolerance by the improper maturation of DCs, which leads to improper activation of T-cells. Many cancer immunotherapies aim to break this tolerance and to generate a functional adaptive immune response that is tumour-specific.

1.3 Cancer Treatment

Currently, the treatment of cancer encompasses four major modalities: radiation therapy, chemotherapy, immunotherapy, and surgery. The course of cancer treatment depends on the type of cancer, its location, and its stage. In many cancer patients, a combinatorial approach is used in order to prevent tumour recurrence and achieve cures. In this overview, we will briefly summarize radiation therapy and chemotherapy and largely focus on the latter two treatment modalities.

1.3.1 Radiation Therapy

Radiation therapy uses high-energy radiation to create highly-reactive free radicals that initiate DNA damage. DNA injury is lethal due to the interruption of cell division (reproductive death) and structural degeneration (interphase death). Rapidly dividing tissues show an early response to radiation while slowly proliferating tissues may not manifest injury for months or years. As a result, radiation therapy results in a net higher rate of cancer cell killing than normal cells as cancer cells proliferate more rapidly[24]. If used before surgery (neoadjuvant therapy), radiation aims to shrink the tumor, and if used after surgery (adjuvant therapy), radiation destroys microscopic tumor cells that may have been left behind[24]. It is well known that tumors differ in their sensitivity to radiation treatment. One of the major limitations of radiation therapy is decreased radiosensitivity due to lack of oxygen (hypoxia) in some types of tumours. Oxygen is essential for effective radiation therapy due to its involvement in radiochemical reactions which produce free radicals and mediate DNA damage[25].

1.3.2 Chemotherapy

Chemotherapy is the treatment of cancer with one or more cytotoxic pharmacological agents. Like radiation therapy, it can be used to treat disseminated disease as it is often administered systemically. Chemotherapy can also be administered as a single modality or in combination with surgery or radiation therapy[26]. Traditional chemotherapeutic agents act by killing cells that divide rapidly. Some examples of such agents include cyclophosmamide, cisplatin, gemcitabine, and fluorouracil[26]. Newer anti-cancer drugs, such as monoclonal antibodies, are more targeted towards proteins that are abnormally expressed in cancer cells. One such example incudes Cetuximab, which targets the epidermal growth factor receptor (EGFR) protein and is routinely used in the treatment of colorectal cancer[6].

1.3.3 Immunotherapy

Immunotherapy is a relatively new class of cancer treatment that works to harness the innate powers of the immune system to fight cancer. Because of the immune system's unique properties, these therapies may hold greater potential than current treatment approaches to fight cancer more powerfully, offer longer-term protection against the disease, come with fewer side effects, and benefit more patients with more cancer types.

1.3.3.1 Non-Targeted Cancer Immunotherapy

Non-specific cancer immunotherapies are those that seek to generally augment various aspects of the immune system to lead to a greater anti-tumour immune response. This includes the use of immune stimulatory cytokines, like IL-2 and IFNα, which are already

in use in the clinic[27]. Other cytokines are in clinical testing, like GM-CSF and IL-21, which seek to enhance APC and T cell activation, respectively. However, all of these treatments induce harsh side-effects that, in some cases, can limit treatment use[27]. As mentioned previously, monoclonal antibodies are also widely used as targeted cancer therapeutics.

1.3.3.2 Targeted Cancer Immunotherapy: Cancer Vaccines

Cancer vaccines present an innovative approach to cancer management. They exert an antitumor effect by engaging the host immune response, and have great potential for circumventing the intrinsic drug resistance that limits standard cancer management. In addition, cancer vaccines are advantageous in that they can be highly specific, have low toxicity, and provide long-lasting treatment efficacy due to immunologic memory[28].

A wide variety of cancer vaccines have been and are being evaluated in clinical trials. Adjuvanted peptide, DNA, mRNA, cell- and vector-based vaccines are all under investigation for the treatment of virtually all cancer types[29-35]. These clinical trials suggest that cancer vaccines can be safe and successfully elicit both detectable and sometimes efficacious immune responses. While cancer vaccines in clinical trials have generally been successful in inducing immune responses, a common problem appears to be that these responses are often low-magnitude, presumably limiting efficacy. A number of recent clinical trials have tested the ability of viral vectors to induce antigen-specific anti-tumour immune responses[36-40]. These trials demonstrated the induction of detectable antibody and cellular immune responses. There were also clear indications of stabilized disease but very few

examples of tumour regressions, indicating that viral vectored tumour vaccines hold promise but require considerable optimization.

In this regard, additional consideration must be given to the timing and sequence of standard therapies (including surgery and chemotherapy) relative to the vaccine schedule, and using a strategy that combines non-specific immunomodulators with the cancer vaccine to optimize efficacy.

1.3.4 Surgery

Surgery is a necessary and crucial intervention to treat solid malignancies in cancer patients. However, previous research demonstrated by the Auer lab and others have shown that surgery can accelerate the growth of pre-existing metastases and promote the metastatic potential of tumour cells[41-44]. The acute and major immunosuppression that occurs following surgery has been linked to this phenomenon and there are numerous mechanisms that have been attributed to perioperative metastases. These include the dissemination of tumour cells into the circulation[45], local and systemic release of pro-tumourigenic growth factors and cytokines such as EGF, VEGF, TGFβ, PDGF, IL-6, IL-1β, and IL-10[46], the promotion of angiogenesis through the inhibition of anti-angiogenic factors such as angiostatin and endostatin[47], and the inhibition of cell-mediated immunity (CMI)[48].

Many of the cells and growth factors involved in wound healing are also implicated in tumourigenesis. During wound healing, EGF is released from platelets to initiate mitosis and migration of various types of cells. In several experimental studies, EGF has been shown to increase the metastatic potential of tumour cells co-cultured with normal

fibroblasts[49,50]. The secretion of TGF-α by macrophages, keratinocytes, and platelets accelerates wound healing[51]. Many cancer cells, such as breast carcinoma cells, secrete TGF-α as they proliferate[52]. Another important and well-known player in both wound healing[53] and tumour development is TGF-β, which has capabilities to alter the metastatic potential of tumour cells[54,55]. It is released in wound healing to induce the synthesis of collagen and stimulate extracellular matrix synthesis..

There have been numerous studies that show alterations in immune system function after surgery. Following surgical procedures involving general anaesthesia and tissue injury, activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system is thought to suppress the immune system. Experimental studies by our group and others demonstrate that the suppression of NK cell activity after surgery has been shown to promote tumour development[56]. In humans, surgical procedures for localized tumour resection have led to the suppression of NK cell activity during the post-operative period. Specifically, defects in NK cell lysis and diminished responses to recombinant IFNγ have been observed[57-59]. In addition to changes in NK cell activity, this clinical study also observed that T-cell proliferation was decreased after surgery[59].

Catecholamines, which are released after tissue injury, have also been found to suppress NK cell activity[60]. After tissue injury, norepinephrine (NE), which is a catecholamine, is released into the peripheral circulation[61]. Macrophage function has been shown to be inhibited by NE by adrenergic receptors[62]. Suppression of macrophage function could inhibit initiation of the adaptive immune response.

There have been many studies suggesting alteration in the number of immune cells following surgery. Importantly, not only were lymphocytes shown to decrease in numbers following surgery, but their capability to proliferate and produce cytokines was impaired as well[63]. Furthermore, reduced post-operative expression of HLA-DR, which is the MHC class II molecule in humans, on monocytes possibly indicate that antigen presentation is impaired following surgery[50,64].

Although the initial postoperative cytokine response is pro-inflammatory at the site of injury, induction of a systemic anti-inflammatory response ensues, which in turn cause cellular immune suppression. This anti-inflammatory response is thought to restrict inflammation to the site of injury, prevent inflammatory damage to other tissues and organs, and limit unwanted systemic immune reactions[65]. This systemic immune suppression after surgery could aid the growth of disseminated disease by thwarting an active immune response.

Together, these studies suggest that suppression of immune functions or facilitation of tumour growth may increase the susceptibility to tumour metastasis during or shortly after surgery. As surgery is the primary treatment modality for most solid tumours, the long-term outcome after cancer surgery could be affected.

1.4 Rationale

The study of surgical stress and subsequent increased perioperative metastases has largely focused on the impairment of innate immune cells such as natural killer (NK) cells[66]. Although perioperative lymphopenia and dampened T-cell activity following surgery are

well reported[63,67,68], the impact of surgery on tumour antigen-specific adaptive immunity is poorly understood. This important and clinically relevant question begs clarification considering cancer treatment using vaccination is an exciting therapeutic approach that is being actively pursued[69,70]. Moreover, cancer vaccines are best suited to eradicate minimal residual disease (MRD)[71-73], providing a strong rationale to combine surgery and immunotherapy. Among these vaccination strategies include viral vectors expressing TAAs that can elicit strong tumour-specific humoral and cell-mediated immune responses and consequently result in tumour regression and improved survival[74-76].

One such viral vector-based tumour vaccine is AdhDCT, a replication-incompetent E1/E3-deleted human type 5 adenovirus that expresses the full-length human dopachrome tautomerase (hDCT) gene[77]. Also known as tyrosine related protein-2 or Trp-2, DCT is a protein involved in melanogenesis and is present in both normal melanocytes and melanomas[78]. It is an attractive, clinically relevant model antigen for immunotherapy studies because it can be recognized by melanoma-reactive cytotoxic T lymphocytes (CTLs) in both C57BL/6 mice and human melanoma patients[79]. Moreover, the immunodominant peptide recognized by these CTLs is shared by both murine and human analogs of DCT.

The DCT-specific T-cell responses (both CD4+ and CD8+ T-cells) following prophylactic and therapeutic AdhDCT administration both in tumour-naive and B16F10 melanoma mouse models are well characterized[77,80-82]. It has been previously shown that prophylactic AdhDCT immunization can confer protection against B16F10 melanoma in a CD8-dependent manner[82].

In addition, previous studies in our lab have suggested that a robust anti-tumour immune response generated against an established CT26lacZ murine colorectal tumour was significantly abrogated by surgery and this protection was also dependent on cytotoxic CD8+ T-cells (Kelley Parato and Agnieska Kus unpublished data).

Therefore, in the present study, we focused our attention on the effect of surgical stress on tumour-specific cytotoxic T lymphocytes (CTLs) in an AdhDCT-immunized B16 tumour model. We hypothesize that surgical stress attenuates tumour-specific CD8+ T-cells in immunized mice.

1.5 Objectives

1. Develop a vaccine model of B16 melanoma to illustrate the effect of surgery on acquired anti-tumour (DCT-specific) immunity
2. Characterize the perioperative tumour-specific T-cell immune response in a vaccine model of B16
3. Demonstrate the importance of surgical stress in a clinically relevant B16 surgery model of minimal residual disease
4. Rescue surgery-induced abrogation of tumour-specific T-cells using perioperative immune-stimulating therapeutics

2 - MATERIALS AND METHODS

2.1 Animals and cells

Female age-matched (6–8 wk old at study initiation) C57BL/6 and CD-1 nude mice (Charles River Laboratories, Wilmington, MA) were housed in specific pathogen-free conditions. Isofluorane was used for anesthesia. Animal studies complied with Canadian Council on Animal Care guidelines and were approved by the University of Ottawa's Animal Research Ethics Board. B16F10 melanoma cells were purchased from the American Type Culture Collection. B16F10-LacZ cells were a gift from Dr. Anne Chambers. Cell lines were propagated in Dulbecco's Modified Eagle Medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1x of Penicillin/Streptomycin (Invitrogen).

2.2 Viruses and reagents

AdhDCT was provided by Dr. Brian Lichty (McMaster University). AdhDCT expresses the full-length human DCT protein. Adenovirus was propagated in 293 cells and purified on a cesium chloride gradient as previously described[83].

2.3 Immunization protocol

Anesthetized mice were immunized with 1×10^6 or 1×10^7 PFU AdhDCT (reconstituted in 100µl PBS) by intramuscular (IM) injection into each thigh (50µl per hamstring). Control mice received PBS.

2.4 Generation of surgical stress

To induce surgical stress, anesthetized mice underwent full abdominal laparotomy (4-5cm incision) and left nephrectomy (denoted as Nx in Figures). For pain management, mice receive 3 doses of buprenorphine on the day of surgery and 2 doses per day of buprenorphine for 2 more days.

2.5 Intravenous B16 model

In systemic dissemination models, mice were challenged with $1x10^6$ B16F10-LacZ cells administered IV in the tail vein immediately prior to surgery, and 7 days after AdhDCT vaccination. Mice were culled 3 days later and tumour-bearing lungs were stained with X-gal to visualize tumour micrometastases.

2.6 Subcutaneous B16 model

Subcutaneous (SQ) melanoma tumours were established by injecting $1x10^5$ B16F10 or $3x10^5$ B16F10-LacZ cells in serum-free media in the right hind flank of the mouse; tumour size was measured twice a week and tumour volumes were calculated using the formula $1/2(L \times W^2)$. In the prophylactic immunization models, cells were injected immediately prior to surgery, and 7 days after AdhDCT vaccination. In the therapeutic immunization model of minimal residual disease, mice were injected SQ with $1x10^5$ B16F10 cells in serum-free media in the right hind flank on day 0. On day 7, mice were treated with AdhDCT or PBS. On day 14, SQ tumours were resected with a 2x2mm positive margin (Res), with half receiving additional surgical stress in the form of abdominal laparotomy and left nephrectomy (Res + Nx).

2.7 Adoptive T-cell transfer

C57/BL-6 mice (n=10/group) were prophylactically immunized with AdhDCT or PBS and underwent surgery or no surgery as previously described. Mice were endpointed 24 hours after surgery and pan T-cells were isolated from the spleen using a CD3 MicroBead kit as per manufacturer's instructions (Miltenyi Biotec). $10x10^6$ CD3+ T-cells were transferred IV into naive recipient mice (n=5/group) and 24 hours later, these mice were challenged with SQ $3x10^5$ B16F10-LacZ cells in the right hind flank. Tumour growth was monitored.

2.8 Peptides

The immunodominant peptide from DCT that binds to H-2Kb ($DCT_{180–188}$, SVYDFFVWL; shared by human and murine DCT) was synthesized by Biomer Technology (Pleasanton, California).

2.9 Isolation of spleen lymphocytes

Spleens were processed by mashing with a syringe plunger. Released cells were filtered through a 70µM strainer and red blood cells (RBCs) were removed with ammonium-chloride-potassium (ACK) lysis buffer. For peptide restimulation, cells were reconstituted in Roswell Park Memorial Institute (RPMI) medium (HyClone) supplemented with 10% FBS (HyClone) and 1x of Penicillin/Streptomycin (Invitrogen). For flow cytometry, cells were reconstituted in fluorescence-activated cell sorting (FACS) buffer.

2.10 In vitro peptide restimulation and intracellular flow cytometry

1-2x10^6 spleen cells were stimulated with peptides (2µg/ml), or PMA (0.1µg/mL) and ionomycin (1µg/mL), in the presence of brefeldin A (GolgiPlug; BD Pharmingen, 1µg/ml added after 1.5 hours of incubation). After 6 hours of total incubation time, cells were treated with anti-CD16/CD32 and cell surface antigens were labeled with fluorochrome-conjugated antibodies. Cells were then permeabilized and fixed with Cytofix/Cytoperm (BD Pharmingen) and stained for intracellular cytokines. Data was acquired using a Cyan-ADP 9 flow cytometer with Summit 4.3 software (Beckman Coulter) and analyzed with Kaluza 1.2 software (Beckman Coulter).

2.11 Mouse IFNγ enzyme-linked immunospot (ELISpot) assay

Spleen cells were labelled with Mouse CD8α (Ly-2) MicroBeads (Miltenyi Biotec) and separated using automated magnetic-associated cell sorting (autoMACS Pro Separator; Miltenyi Biotec). For the assay, 2×10^5 CD8α+ T-cells were stimulated with $DCT_{180-188}$ peptide (2µg/mL) and incubated in a microtitre plate coated with IFNγ capture antibody (Mabtech #3321-4AST-4). After 24 hours at 37°C, cytokine secretion was detected as per manufacturer's instructions.

2.12 T-cell apoptosis assay

Isolated splenocytes were treated with anti-CD16/CD32 and cell surface antigens were labeled with PE-CD3 and FITC-CD8α (eBioscience). Cells were then labelled with APC-AnnexinV and 7-AAD (BD Pharmingen) for 15 minutes and data was acquired within an hour.

2.13 BrdU T-cell proliferation assay

Isolated splenocytes were restimulated in vitro with peptides (2μg/ml) at 37°C in the presence of brefeldin A (GolgiPlug; BD Pharmingen, 1μg/ml added after 1.5 hours of incubation). After 3.5 hours of incubation, cells were labelled with BrdU (BD Pharmingen) and incubated for an additional hour. After 6 hours of total incubation time, cells were treated with anti-CD16/CD32 and cell surface antigens were labeled with PerCP-CD3 and PE-CD8α. Following manufacturer's instructions, cells were treated through a series of permeabilizations and fixations and stained for intracellular FITC-BrdU.

2.14 Tumour-infiltrating lymphocyte (TIL) isolation from matrigel

Following the regular prophylactic immunization schedule, mice were challenged with 3x105 B16-F10 cells resuspended in 300uL of matrigel (BD Biosciences). Three days after tumour challenge, mice were euthanized. Six hours before euthanasia, mice were treated IV with 0.25mg Brefeldin A (Sigma), as previously published(45). Mice were euthanized and matrigel plugs were excised from the flank, cut into approximately 1-3mm pieces, and disaggregated for 1-2 hours using a cocktail of collagenase type IV (Cooper Biomedical), Dispase, and DNase I (Invitrogen) resuspended in HBSS at 37˚C. This mixture was then washed and stained as described above.

2.15 Statistical analyses

Statistical tests were performed using GraphPad Prism (GraphPad 4.0). Means of two groups were compared using two-tailed paired Student's t-test. When more than two

groups were compared, one-way ANOVA and Bonferroni multiple comparison post-hoc analysis was conducted. For survival studies, statistical significance was evaluated using Kaplan–Meier survival curves with log-rank tests.

3 – RESULTS

3.1 Surgical stress increases post-operative lung metastases in mice immunized prophylactically with AdhDCT.

Previous work by Agnieszka Kus demonstrated that a robust anti-tumour immune response generated against an established CT26lacZ murine colorectal tumour by multi-dose oncolytic VSVd51 therapy was significantly abrogated by surgery, resulting in the outgrowth of a secondary flank tumour challenge. Therefore, we wanted to determine if surgical stress could attenuate pre-existing anti-tumour immunity against a known clinically-relevant tumour antigen. Previous studies demonstrate that AdhDCT is extremely effective against B16F10 melanoma when administered prophylactically and its anti-tumour efficacy significantly diminishes even if administered 1 day after tumour cell inoculation. Furthermore, previous characterization of the CD8+ T-cell response following AdhDCT immunization revealed that the peak of the effector CD8+ T-cell response occurs between 7-10 days.

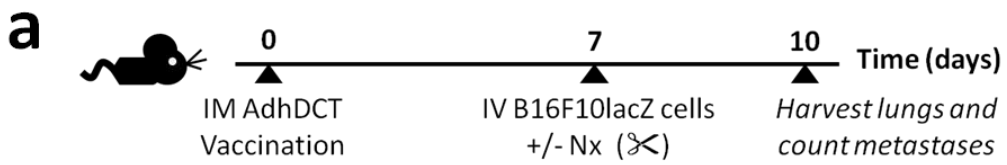
Our group has previously shown that when tumour cells are injected intravenously immediately before surgery, there is a significant increase in perioperative lung micrometastases when quantified three days following surgery[41]. Thus, we decided to assess the impact of surgical stress on the DCT-specific T-cell immune response conferred

by prophylactic AdhDCT immunization in our existing tumour dissemination model of surgical stress (**Figure 1**). For timing, the AdhDCT vaccine was administered 7 days prior to cell injection and surgery to overlap the peak effector CD8+ T-cell response with the induction of surgical stress (**Figure 1a**).

Mice immunized with saline (PBS) that underwent major abdominal surgery developed 200% more lung micrometastases than their non-surgery counterparts. Mice immunized with 1×10^6 pfu AdhDCT were protected against B16lacZ tumour challenge and demonstrated 300% reduction of lung micrometastases compared to PBS-treated controls. In contrast, when AdhDCT-immunized mice were surgically-stressed, they developed 500% more lung micrometastases compared to AdhDCT-immunized mice that did not undergo surgery (**Figure 1b,c**).

3.2 Perioperative anti-Asialo (GM1) depletion in immunized mice suggests clearance of lung metastases is primarily mediated by NK cells

Previous studies of AdhDCT immunization have shown that the robust anti-tumour response conferred by the vaccine is primarily due to T-cells. On the other hand, our group has shown that surgical stress attenuates NK cell function and in IL-2 receptor gamma-knockout (IL-2rg-KO) mice (which are T-cell deficient), surgical stress led to a prometastatic effect[41]. This suggests that the clearance of intravenously-injected tumour cells is primarily NK cell-mediated, albeit these experiments were conducted in a non-immunized context. Therefore, we depleted NK cells in this model to determine if the prometastatic effect of surgery in AdhDCT-immunized mice was primarily due to surgery-induced dysfunction of NK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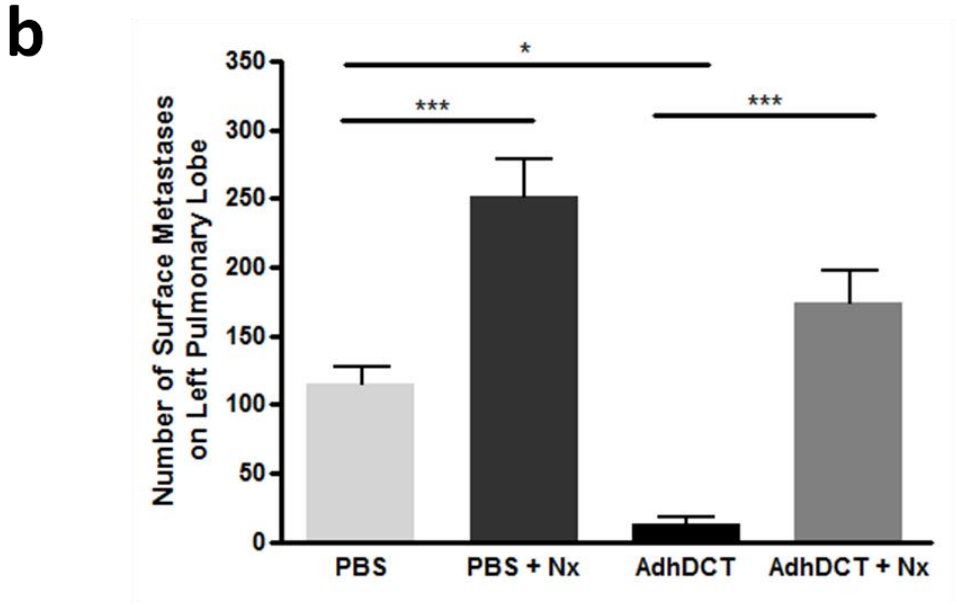
IM AdhDCT Vaccination
IV B16F10lacZ cells +/- Nx (✂)
Harvest lungs and count metastases


b
Number of Surface Metastases on Left Pulmonary Lobe
350
300
250
200
150
100
50
0
*
***
***
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

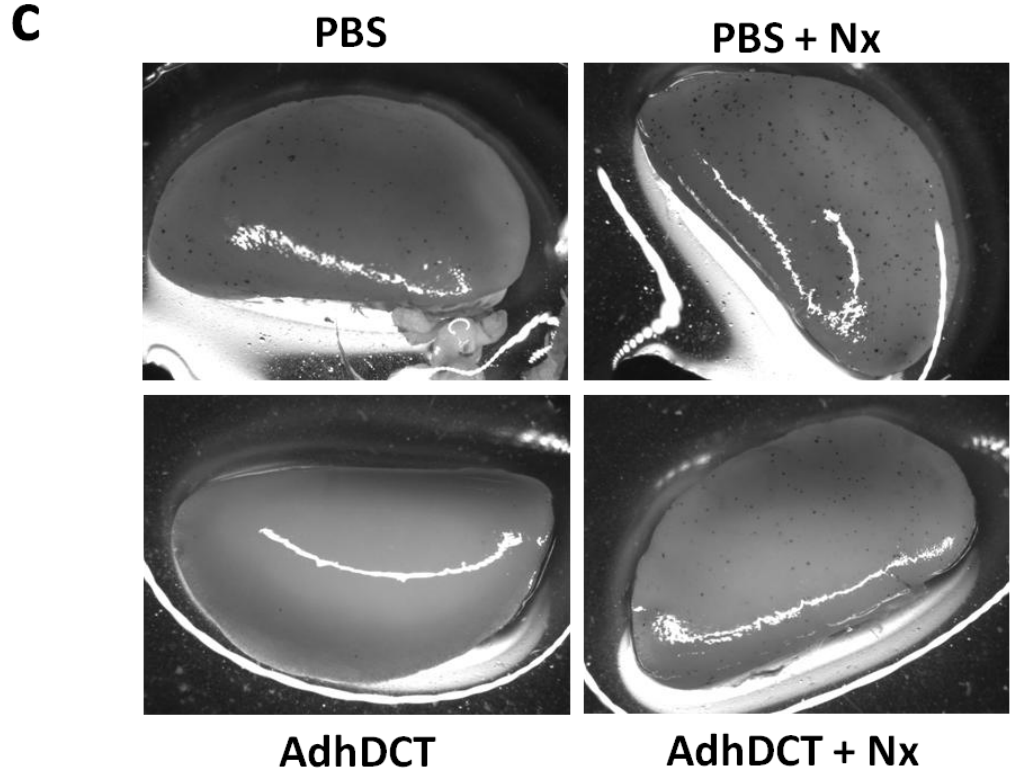

c
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

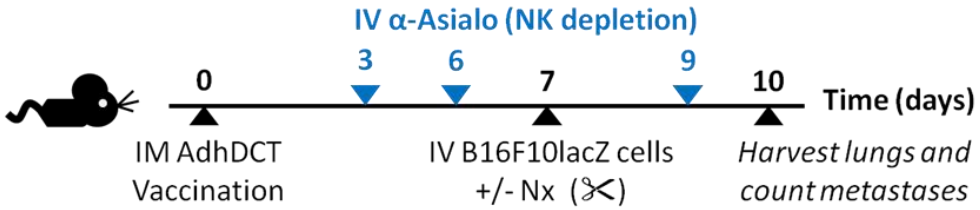
Figure 1. Lung metastases following prophylactic AdhDCT immunization and intravenous B16F10-LacZ tumour challenge in surgically-stressed mice. a) Experiment scheme. On day 0, C57/BL-6 mice (n=5-6/group) were injected IM with PBS or $1x10^6$ pfu AdhDCT. On day 7, mice were injected IV with $1x10^6$ B16F10-LacZ cells. Following cell injection, mice were anesthetized and either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 10, mice were euthanized and lungs were harvested and stained with X-Gal to enumerate lung metastases. **b)** Pulmonary surface metastases on the left lobe were counted using a dissecting microscope and ImageJ. **c)** Representative pictures of lung micrometastases. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis.*p<0.05, **p<0.01, ***p<0.001

cells as opposed to dysfunction of T-cells (**Figure 2**). NK cells were depleted using multiple injections of anti-asialo-GM1 antibody around the time of surgery (**Figure 2a**). Analysis of lung micrometastases on day 10 illustrate that the tumour burden in AdhDCT-immunized mice is similar to saline-treated control animals (**Figure 2b,c**). This suggests that even in the context of immunization, clearance of tumour cells injected intravenously is mediated by NK cells.

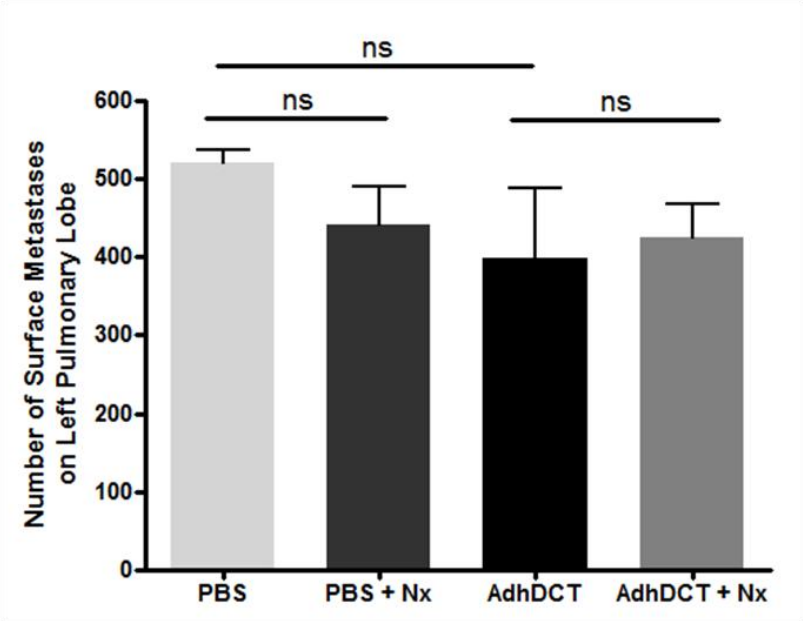
3.3 Prophylactic AdhDCT immunization confers protection against subcutaneous B16 tumour outgrowth and is abrogated by surgical stress

Since the lung metastases model may be biased towards NK cell-mediated tumour clearance, we proceeded to investigate DCT-specific immunity in a subcutaneous B16 tumour challenge model of surgical stress. As in the previously described model, mice were immunized with 1×10^6 pfu AdhDCT or PBS one week prior to B16F10lacZ cell injection and surgery and tumour outgrowth was monitored until a 15mm diameter was reached (**Figure 3a**). Interestingly, we found that 100% of mice immunized with AdhDCT did not develop tumours, whereas 100% of mice that underwent surgery eventually developed and

a


IV α-Asialo (NK depletion)
3
6
9
0
7
10
Time (days)
IM AdhDCT
Vaccination
IV B16F10lacZ cells
+/- Nx (✂)
Harvest lungs and
count metastases

b


ns
ns
ns
Number of Surface Metastases
on Left Pulmonary Lobe
600
500
400
300
200
100
0
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

c

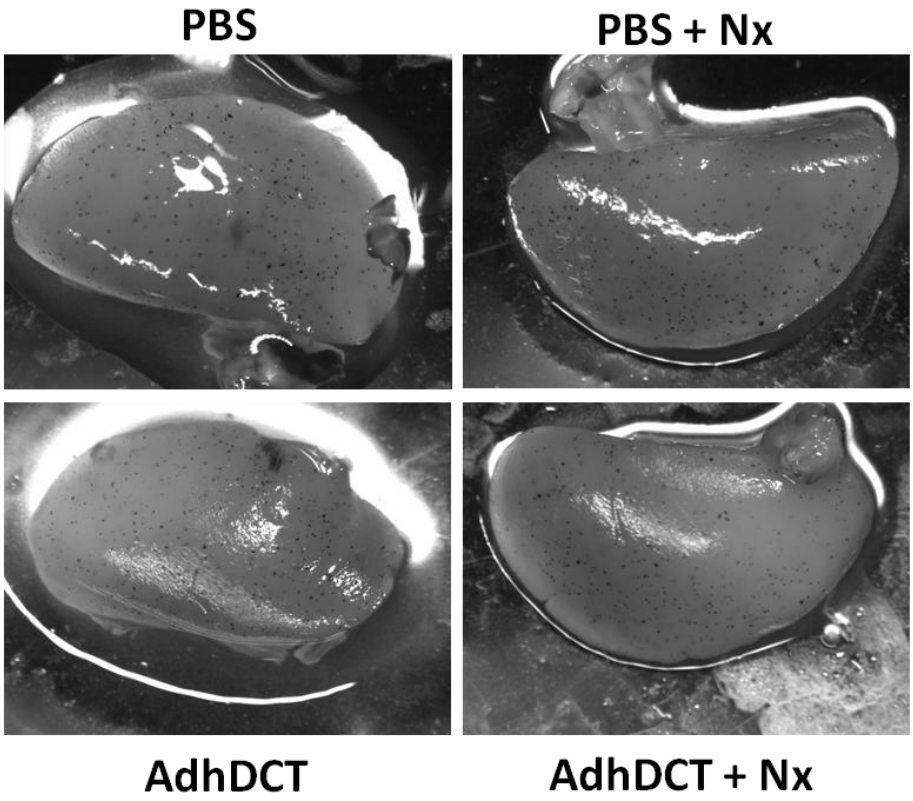

PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

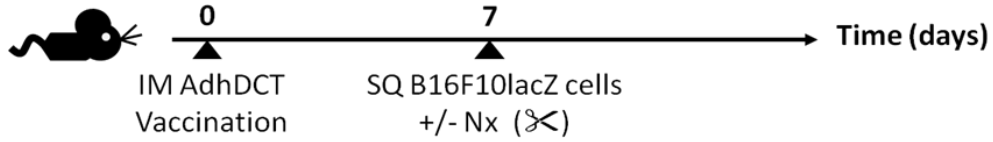
Figure 2. Lung metastases following perioperative anti-Asialo NK cell depletion and intravenous B16F10-LacZ tumour challenge in surgically-stressed mice. a) Experiment scheme; on day 0, C57/BL-6 mice (n=4-5/group) were injected IM with PBS or 1x106 pfu AdhDCT; on days 3, 6, and 9, NK cells were pharmacologically depleted by IV injection of 50µl of anti-Asialo antibody; on day 7, mice were challenged IV with 1x106 B16F10-LacZ cells; following cell injection, mice were anesthetized and either underwent full abdominal laparotomy and left nephrectomy surgery (Nx) or did not; on day 10, mice were euthanized and lungs were harvested and stained with X-Gal to enumerate lung metastases. **b)** Pulmonary surface metastases on the left lobe were counted using a dissecting microscope and ImageJ. **c)** Representative pictures of lung micrometastases. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis. ns=not significant ($p>0.05$)

succumbed to their tumours (**Figure 3b**). To determine if this trend was due to the high immunogenicity of the trangene-encoding B16F10LacZ cell line, we performed the experiment using wild-type B16F10 flank challenge and a 10-fold increase in the AdhDCT vaccine dose to accommodate for this cell line's poor immunogenicity and aggressive tumour growth phenotype (**Figure 3c**). To our surprise, we again found that AdhDCT vaccination protected all mice from developing tumours, whereas 100% of AdhDCT-immunized mice that underwent major abdominal surgery developed and succumbed to their tumours (**Figure 3d**).

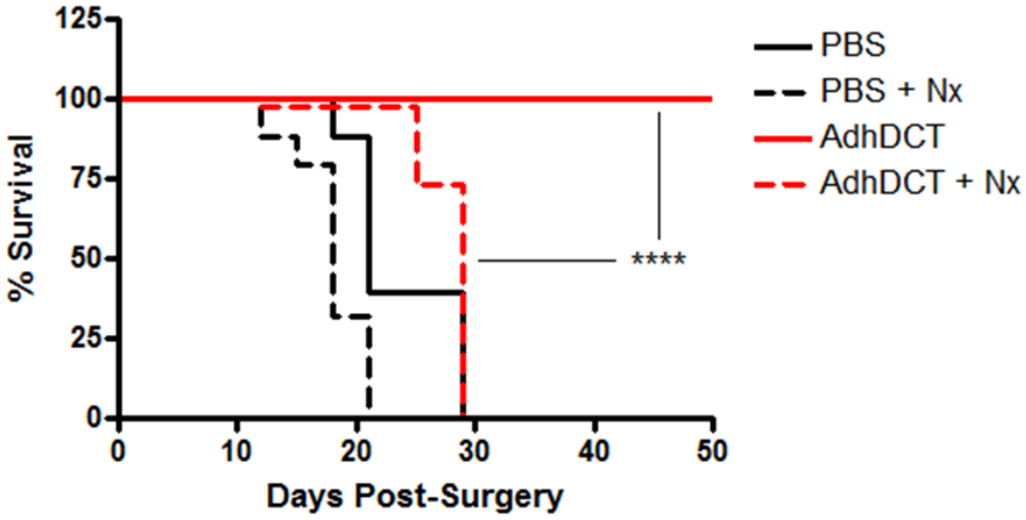
3.4 Protection against subcutaneous B16 tumour outgrowth conferred by AdhDCT vaccination requires T-cells

To determine if there was a mediating role for T-cells in this phenomenon, we replicated the experiment described above in athymic CD-1 nude mice that are deficient in T-cells, but retain NK cell function (**Figure 4a**). In nude mice, AdhDCT does not confer

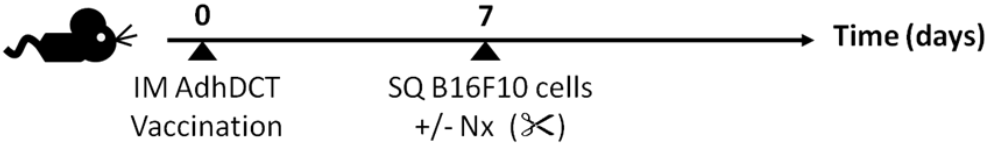
a


0
7
Time (days)
IM AdhDCT
Vaccination
SQ B16F10lacZ cells
+/- Nx (✂)

b


125
100
75
50
25
0
% Survival
0
10
20
30
40
50
Days Post-Surgery
****
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

c


0
7
Time (days)
IM AdhDCT
Vaccination
SQ B16F10 cells
+/- Nx (✂)

d

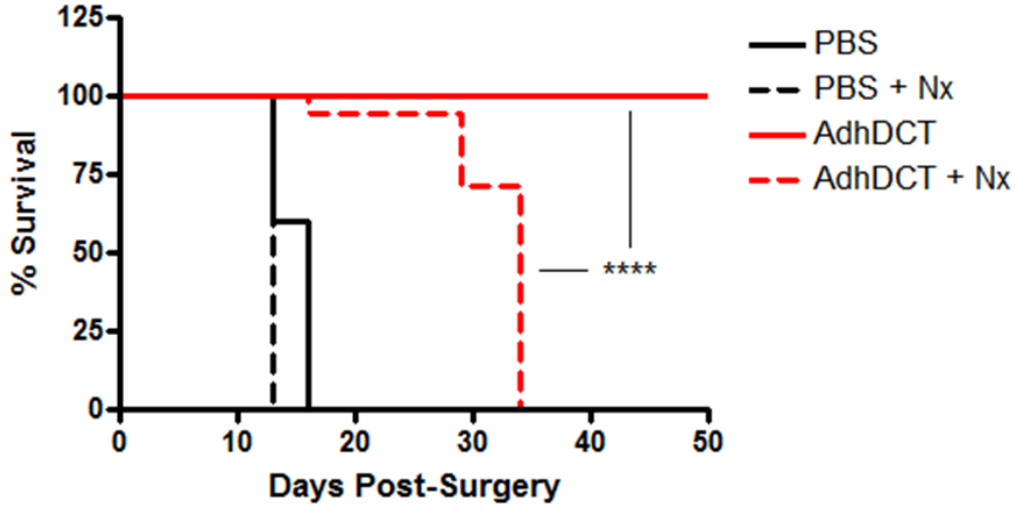
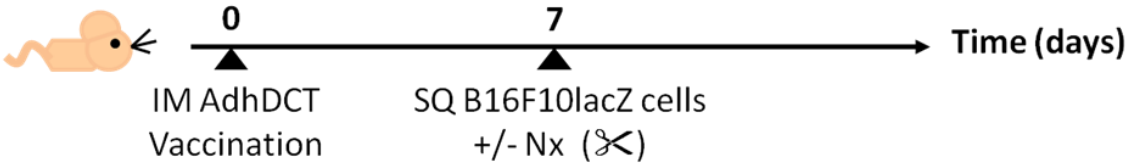

125
100
75
50
25
0
% Survival
0
10
20
30
40
50
Days Post-Surgery
****
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

Figure 3. Prophylactic AdhDCT immunization and subcutaneous B16F10-LacZ tumour challenge survival in surgically-stressed C57/BL-6 mice. a) Experiment scheme; on day 0, C57/BL-6 mice (n=7-8/group) were injected IM with PBS or $1x10^6$ pfu AdhDCT; on day 7, mice were injected SQ with $3x10^5$ B16F10-LacZ cells in right hind flank; following cell injection, mice were anesthetized and either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery; tumour outgrowth was monitored and mice were endpointed when tumour diameter reached 15mm. **b)** Kaplan-Meier survival curves of the treated B16F10-LacZ tumour-bearing mice. **c)** Experiment scheme; on day 0, C57/BL-6 mice (n=7/group) were injected IM with PBS or $1x10^7$ pfu AdhDCT; on day 7, mice were injected SQ with $1x10^5$ B16F10 cells in right hind flank; following cell injection, mice were anesthetized and either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery; tumour outgrowth was monitored and mice were endpointed when tumour diameter reached 15mm. **d)** Kaplan-Meier survival curves of the treated B16F10 tumour-bearing mice. Statistical significance was determined by log-rank tests. **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

a


0
7
Time (days)
IM AdhDCT
Vaccination
SQ B16F10lacZ cells
+/- Nx (✂)

b

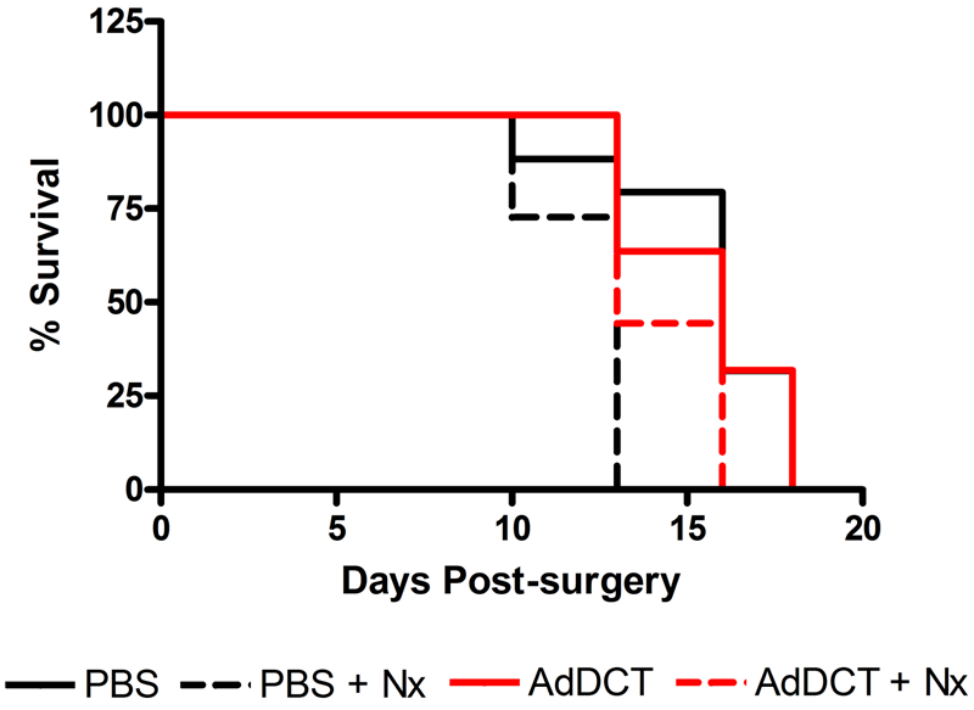

125
100
75
50
25
0
% Survival
0
5
10
15
20
Days Post-surgery
PBS
PBS + Nx
AdDCT
AdDCT + Nx

Figure 4. Prophylactic AdhDCT immunization and subcutaneous B16F10-LacZ tumour challenge survival in surgically-stressed CD-1 nude mice. **a)** Experimental design. On day 0, CD-1 nude mice (n=7/group) were injected IM with PBS or $1x10^6$ pfu AdhDCT. On day 7, mice were injected SQ with $3x10^5$ B16F10-LacZ cells in the right hind flank. Following cell injection, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. Tumour outgrowth was monitored over time and mice were endpointed when tumour diameter reached 15mm. **b)** Kaplan-Meier survival curves of the treated B16F10-LacZ tumour-bearing CD-1 mice. Statistical significance was determined by log-rank tests. No significant differences in survival were found.

protection against the growth of B16lacZ tumours and there are no significant differences in survival (**Figure 4b**) between surgically-stressed and non-surgically stressed AdhDCT-immunized mice. This experiment illustrates that T-cells are essential for anti-tumour immunity and suggests that surgery-induced dysfunction of AdhDCT is at least partially due to an impairment of the adaptive immune system.

3.5 T-cells transferred from surgically-stressed AdhDCT-immunized mice into naive mice retain their defect and do not prevent tumour outgrowth.

Previous work in our lab has shown that NK cells are still functionally impaired even when removed from the surgically-stressed microenvironment and transferred to a naive mouse[42]. Therefore, to investigate whether T-cell impairment by surgery could be transferred to naive mice, we performed an adoptive T-cell transfer experiment (**Figure 5**). C57Bl/6 mice were immunized with AdhDCT, underwent surgery, and culled the next day to harvest spleens and isolate $CD3^+$ T-cells. Subsequently, $10x10^6$ $CD3^+$ T-cells were injected intravenously by tail vein into naive C57Bl/6 mice in a 2:1 donor to recipient ratio. One day

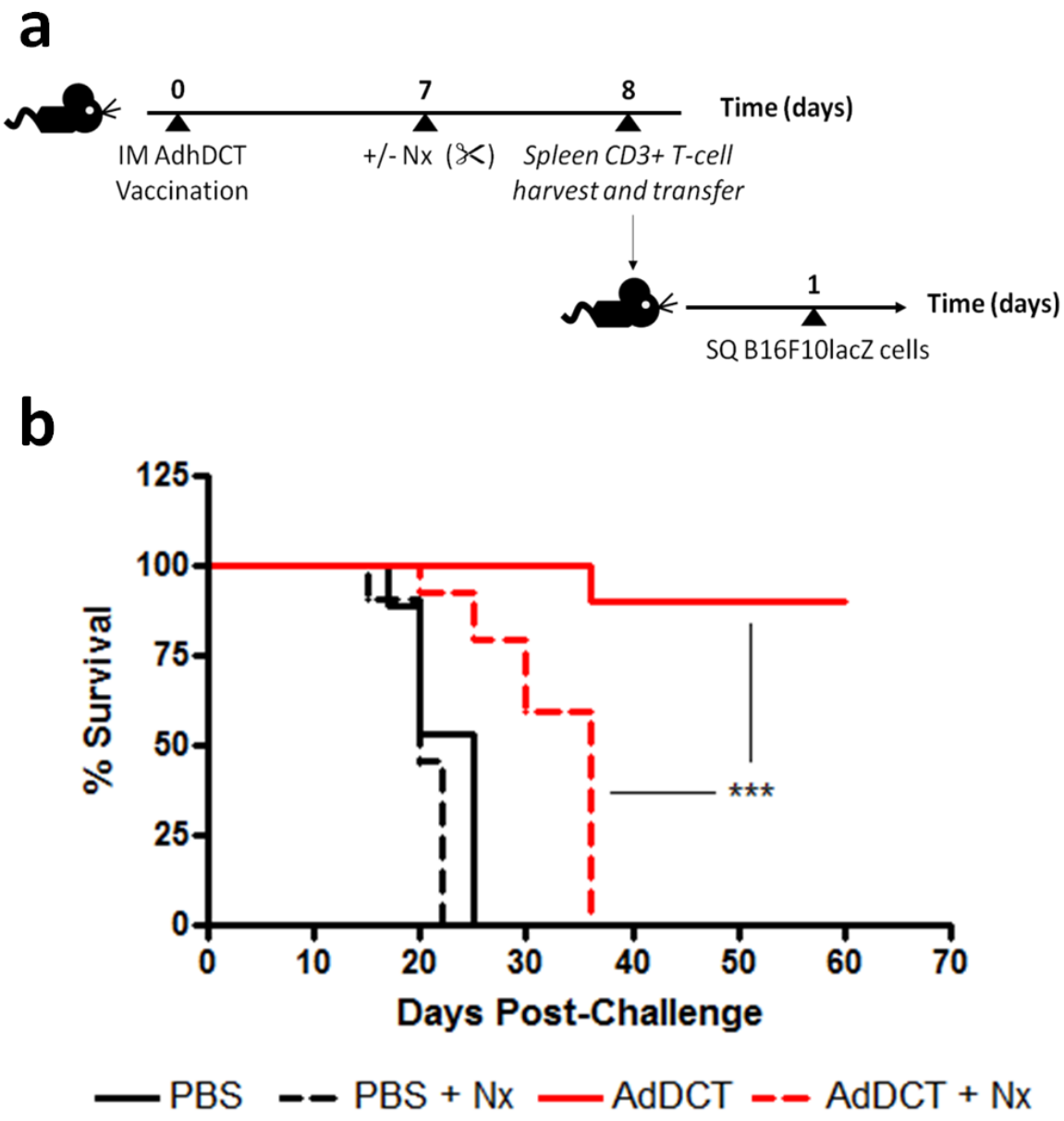

a
0
7
8
Time (days)
IM AdhDCT
Vaccination
+/- Nx (✂)
Spleen CD3+ T-cell
harvest and transfer
1
Time (days)
SQ B16F10lacZ cells
b
125
100
75
50
25
0
% Survival
0
10
20
30
40
50
60
70
Days Post-Challenge
***
PBS
PBS + Nx
AdDCT
AdDCT + Nx

Figure 5. Subcutaneous B16F10-LacZ tumour challenge survival in C57/BL-6 mice following adoptive T-cell transfer from surgically-stressed AdhDCT-immunized mice. a) Experiment design. On day 0, C57/BL-6 mice (n=10/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 8, pan CD3+ T-cells were isolated from the spleen and $10x10^6$ CD3+ T-cells were transferred IV into naive recipient mice (n=5/group). These mice were then challenged 24 hours later with SQ $3x10^5$ B16F10-LacZ cells in the right hind flank. Tumour growth was monitored and mice were endpointed when tumour diameter reached 15mm. **b)** Kaplan-Meier survival curves of the B16F10-LacZ tumour-bearing mice that received adoptive T-cell therapy. Statistical significance was determined by log-rank tests. **p<0.05, **p<0.01, ***p<0.001.*

after T-cells were injected, recipient mice were challenged with SQ B16lacZ tumours on their right hind flanks. Tumour outgrowth and survival was monitored over time (**Figure 5a**). Interestingly, mouse survivals recapitulate the same trends found in our non-transfer experiments. Ninety percent of mice that received T-cells from AdhDCT-immunized mice did not develop tumours, whereas 100% of mice that received T-cells from surgically-stressed mice developed tumours (**Figure 5b**). This indicates that global T-cells taken out of the surgically-stressed environment are still unable to function properly in naive non-surgically stressed mice.

3.6 Surgical stress attenuates the activation of DCT-specific $CD8^+$ T-cells

Next, we sought to explore mechanisms of antigen-specific CD8+ T-cell suppression by surgery. Therefore, we performed a functional assay looking at T-cell activation in response to antigen stimulation with the immunodominant CD8+ T-cell epitope for DCT (**Figure 6a, 7a**). T-cell activation following surgery was assessed by flow cytometry analysis of intracellular cytokines: IFNγ, TNFα, and GranzymeB. One day post-surgery, we observed a

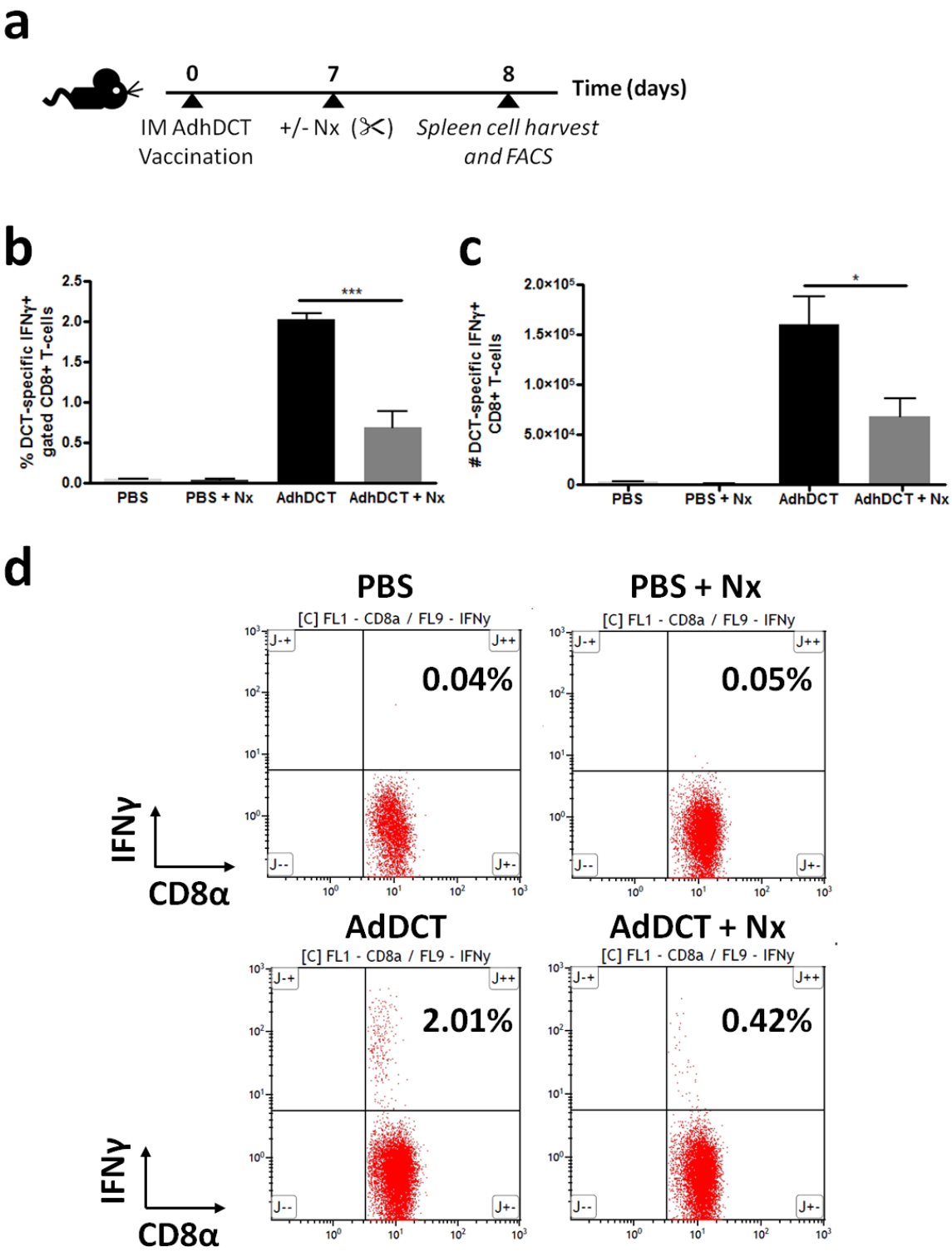

a
0
7
8
Time (days)
IM AdhDCT Vaccination
+/- Nx (✂)
Spleen cell harvest and FACS
b
% DCT-specific IFNγ+ gated CD8+ T-cells
2.5
2.0
1.5
1.0
0.5
0.0
***
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
c
DCT-specific IFNγ+ CD8+ T-cells
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
*
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
d
PBS
[C] FL1 - CD8a / FL9 - IFNy
0.04%
PBS + Nx
[C] FL1 - CD8a / FL9 - IFNy
0.05%
IFNγ
CD8α
AdDCT
[C] FL1 - CD8a / FL9 - IFNy
2.01%
AdDCT + Nx
[C] FL1 - CD8a / FL9 - IFNy
0.42%
IFNγ
CD8α

Figure 6. Frequency and absolute number of $DCT_{180-188}$ peptide-stimulated CD8+ T-cells expressing IFNγ in the spleen 24 hours post-surgery. a) Experiment design. On day 0, C57/BL-6 mice (n=5/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 8, spleen cells were isolated and restimulated in vitro with $DCT_{180-188}$ peptide for 6 hours with GolgiPlug. Cells were then fluorescently labelled and analyzed by FACS. **b)** Proportion of $DCT_{180-188}$-specific CD8+ T-cells expressing IFNγ. **c)** Absolute number of $DCT_{180-188}$-specific CD8+ T-cells expressing IFNγ. **d)** Representative flow dot plots. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis.**p<0.05, **p<0.01, ***p<0.001*

greater than 2-fold decrease in both the frequency (**Figure 6b,d**) and total number (**Figure 6c**) of spleen CD8+ T-cells expressing IFNγ in response to DCT peptide restimulation.

Similarly, AdhDCT-immunized mice demonstrate higher frequencies and total numbers of TNFα (**Figure 7b,c**) and GranzymeB (**Figure 7d,e**) expressing CD8+ T-cells than immunized mice that underwent surgery.

In addition to specific antigen stimulation of T-cells, we also assessed intracellular cytokine production in response to non-specific T-cell activation. In this case, lymphocytes were restimulated with phorbol myristate acetate (PMA) and ionomycin, both mitogens that activate the NFAT (nucelar factor of activated T-cells) transcription factor in T lymphocytes[84]. Again, 24 hours post-surgery (**Figure 8a**), we observed a greater than 2-fold decrease in both the frequency (**Figure 8b,d**) and total number (**Figure 8c**) of spleen CD8+ T-cells expressing IFNγ.

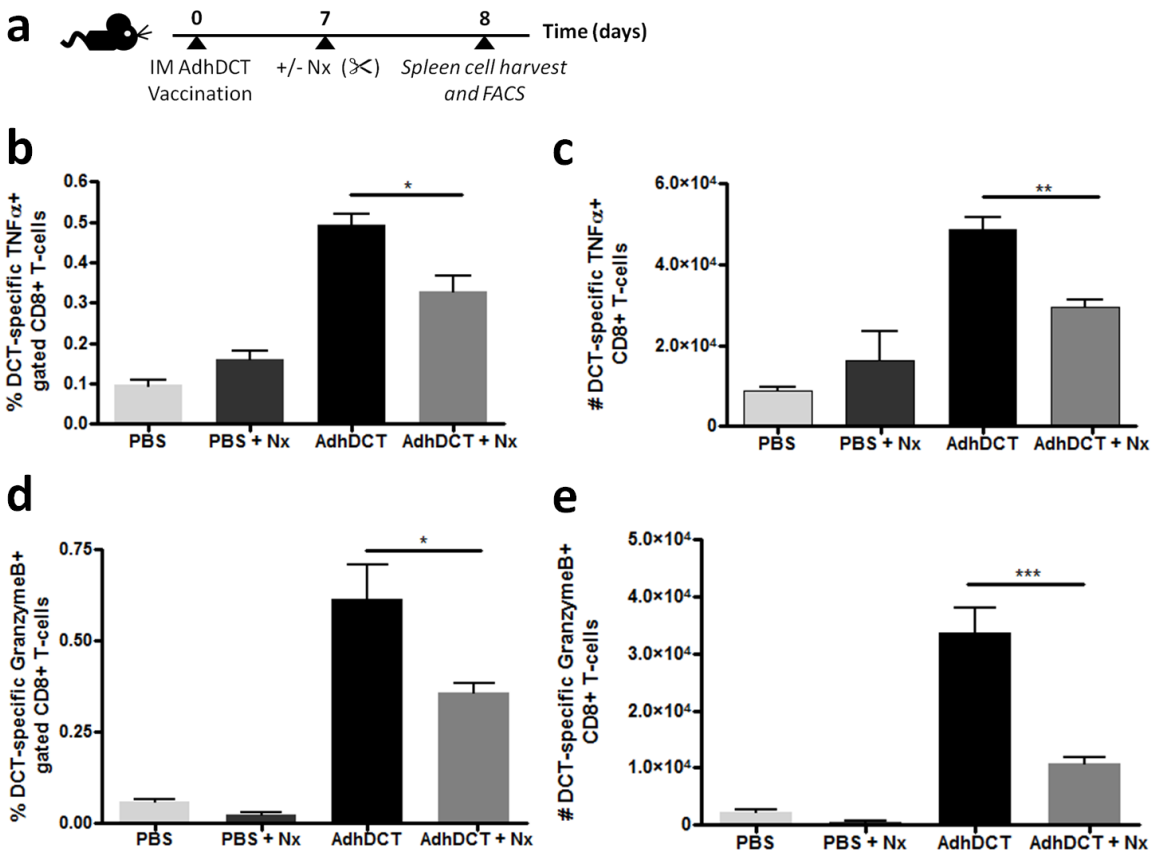

a
0
7
8
Time (days)
IM AdhDCT Vaccination
+/- Nx (✂)
Spleen cell harvest and FACS
b
% DCT-specific TNFα+ gated CD8+ T-cells
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
c
DCT-specific TNFα+ CD8+ T-cells
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
**
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
d
% DCT-specific GranzymeB+ gated CD8+ T-cells
0.75
0.50
0.25
0.00
*
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
e
DCT-specific GranzymeB+ CD8+ T-cells
5.0×10⁴
4.0×10⁴
3.0×10⁴
2.0×10⁴
1.0×10⁴
0
***
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

Figure 7. Frequency and absolute number of $DCT_{180-188}$ peptide-stimulated CD8+ T-cells expressing TNFα and GranzymeB in the spleen 24 hours post-surgery. a) Experiment design. On day 0, C57/BL-6 mice (n=5/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 8, spleen cells were isolated and restimulated in vitro with $DCT_{180-188}$ peptide for 6 hours with GolgiPlug. Cells were then fluorescently labelled and analyzed by FACS. **b)** Proportion of $DCT_{180-188}$-specific CD8+ T-cells expressing TNFα. **c)** Absolute number of $DCT_{180-188}$-specific CD8+ T-cells expressing TNFα. **d)** Proportion of $DCT_{180-188}$-specific CD8+ T-cells expressing GranzymeB. **e)** Absolute number of $DCT_{180-188}$-specific CD8+ T-cells expressing GranzymeB. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis. *$*p<0.05$, $**p<0.01$, $***p<0.001$*

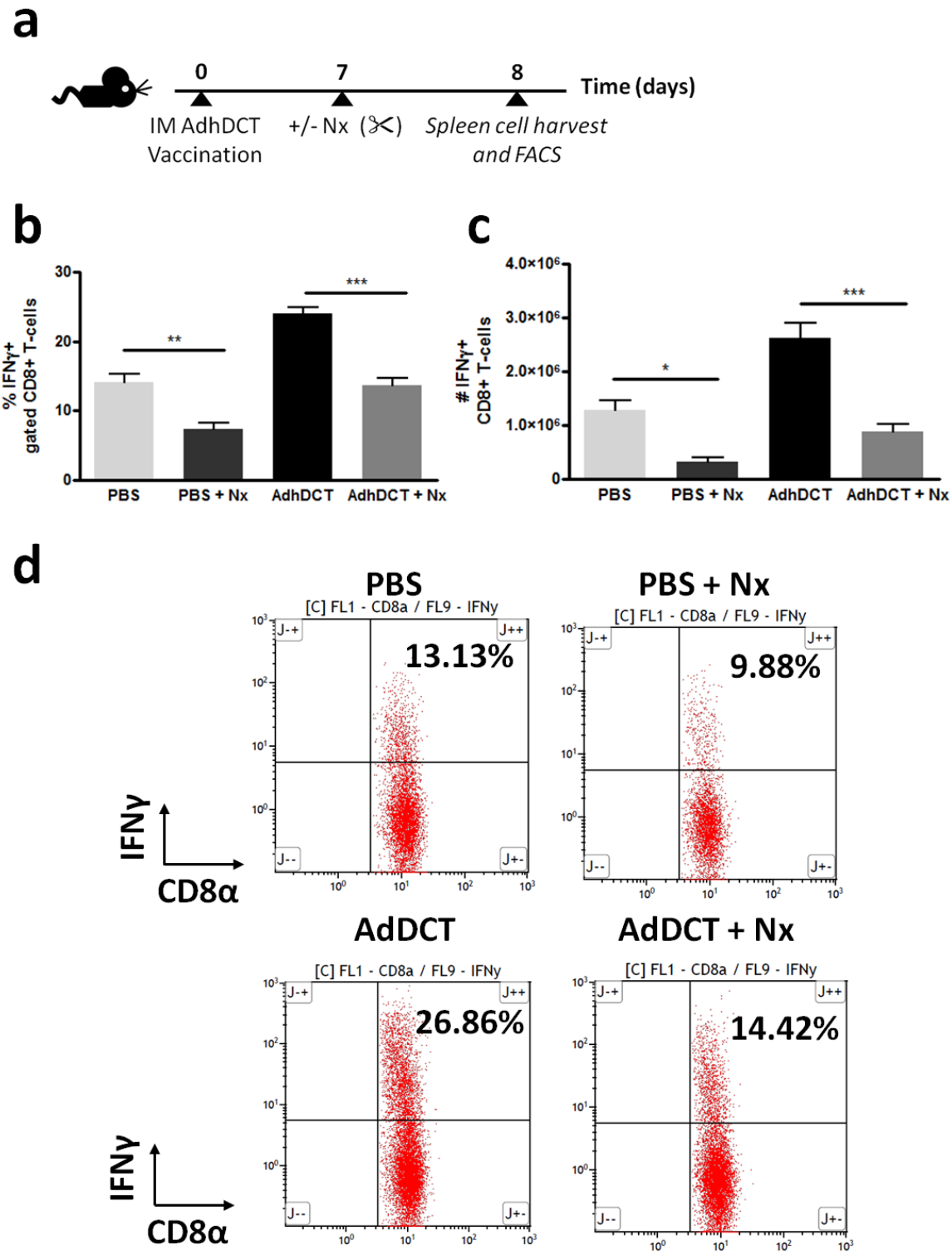

a
0
7
8
Time (days)
IM AdhDCT Vaccination
+/- Nx (✂)
Spleen cell harvest and FACS
b
% IFNγ+ gated CD8+ T-cells
30
20
10
0
**
***
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
c
IFNγ+ CD8+ T-cells
4.0×10⁶
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
*
***
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
d
PBS
[C] FL1 - CD8a / FL9 - IFNy
13.13%
PBS + Nx
[C] FL1 - CD8a / FL9 - IFNy
9.88%
IFNγ
CD8α
AdDCT
[C] FL1 - CD8a / FL9 - IFNy
26.86%
AdDCT + Nx
[C] FL1 - CD8a / FL9 - IFNy
14.42%
IFNγ
CD8α

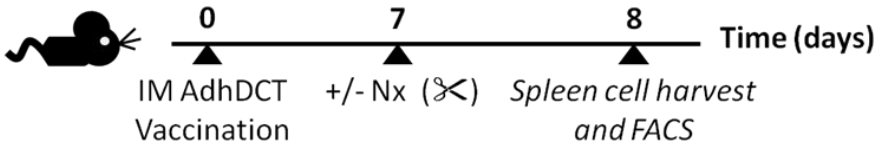
Figure 8. Frequency and absolute number of PMA/ionomycin-stimulated CD8+ T-cells expressing IFNγ in the spleen 24 hours post-surgery. a) Experiment design. On day 0, C57/BL-6 mice (n=5/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 8, spleen cells were isolated and restimulated in vitro with PMA/ionomycin for 6 hours with GolgiPlug. Cells were then fluorescently labelled and analyzed by FACS. **b)** Proportion of CD8+ T-cells expressing IFNγ. **c)** Absolute number of CD8+ T-cells expressing IFNγ. **d)** Representative flow dot plots. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis. **p<0.05, **p<0.01, ***p<0.001*

Furthermore, we also enumerated antigen-specific T-cell responses by IFNγ ELISpot assay to confirm our flow cytometry results (**Figure 9a**). We found a 4-fold attenuation of IFNγ- expression in antigen-specific CD8 T-cells (**Figure 9b,c**) from AdhDCT-immunized mice that underwent surgery.

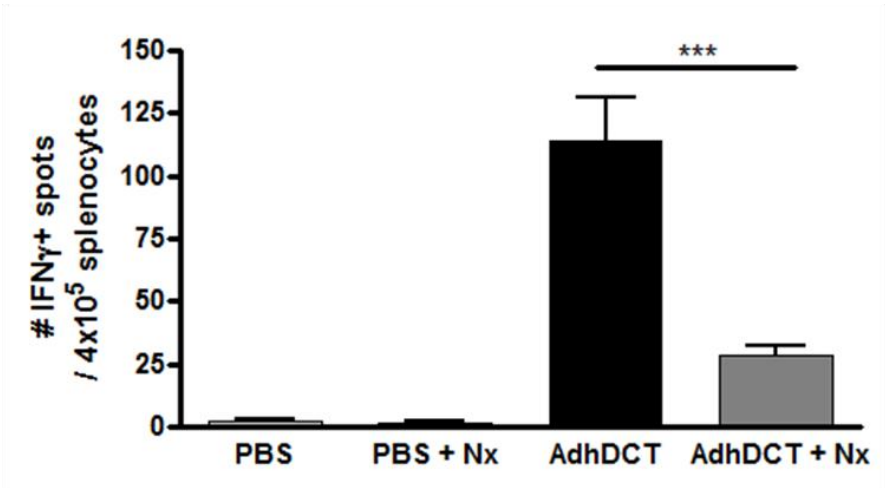
3.7 The effects of surgical stress on T-cell activation are transient and last up to 7 days.

Next, to characterize the duration of surgery-induced T-cell impairment, we performed a kinetic study at different timepoints post-surgery to look at IFNγ expression in DCT-specific CD8+ T-cells (**Figure 10a**). Our results demonstrate that surgical stress attenuates IFNγ expression 1 and 3 days post-surgery (**Figure 10b**). Interestingly, post-operative day 7 appears to be a transition period where recovery starts to occur. By post-operative day 28, the effect of surgical stress on IFNγ production is no longer present. To determine whether recovery of IFNγ expression correlated with tumour survival, a separate cohort of identically treated mice were challenged on day 28 with a subcutaneous B16F10 tumour (**Figure 10c**). However, we observed no differences in tumour growth rates in

a


0
7
8
Time (days)
IM AdhDCT Vaccination
+/- Nx (✂)
Spleen cell harvest and FACS

b


150
125
100
75
50
25
0
IFNγ+ spots / 4x10^5 splenocytes
***
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

c

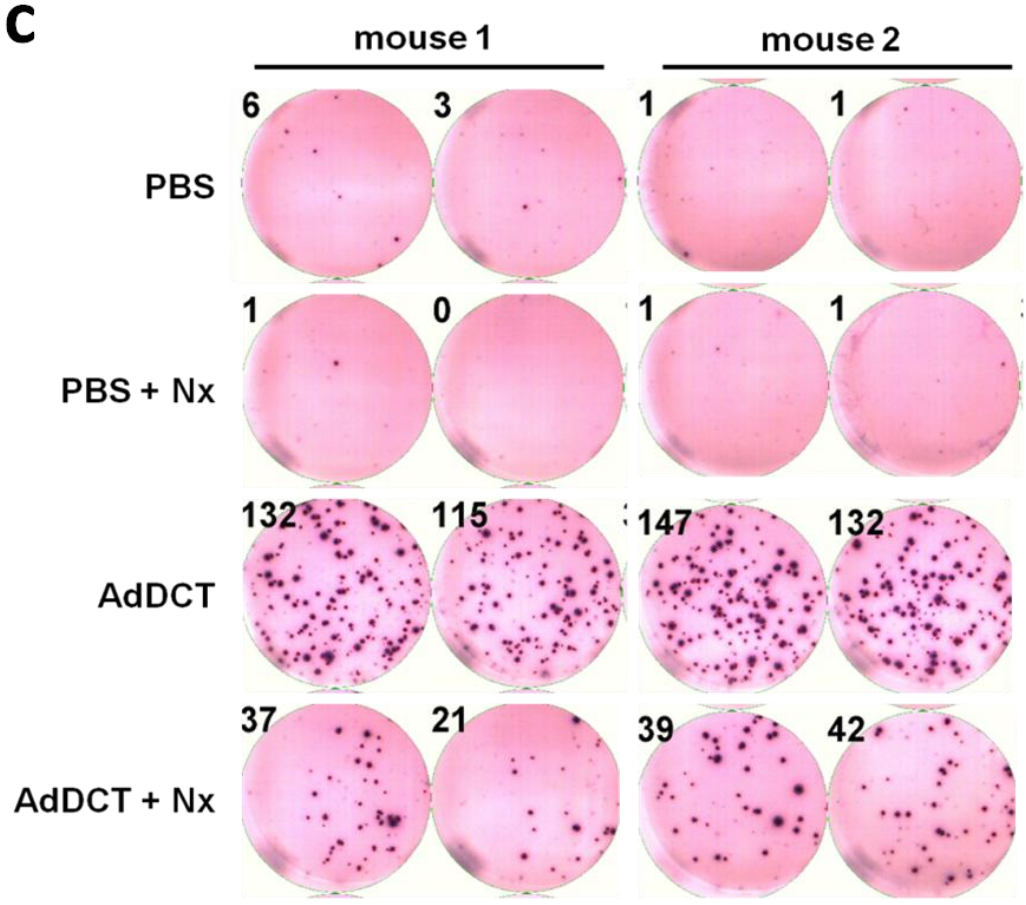
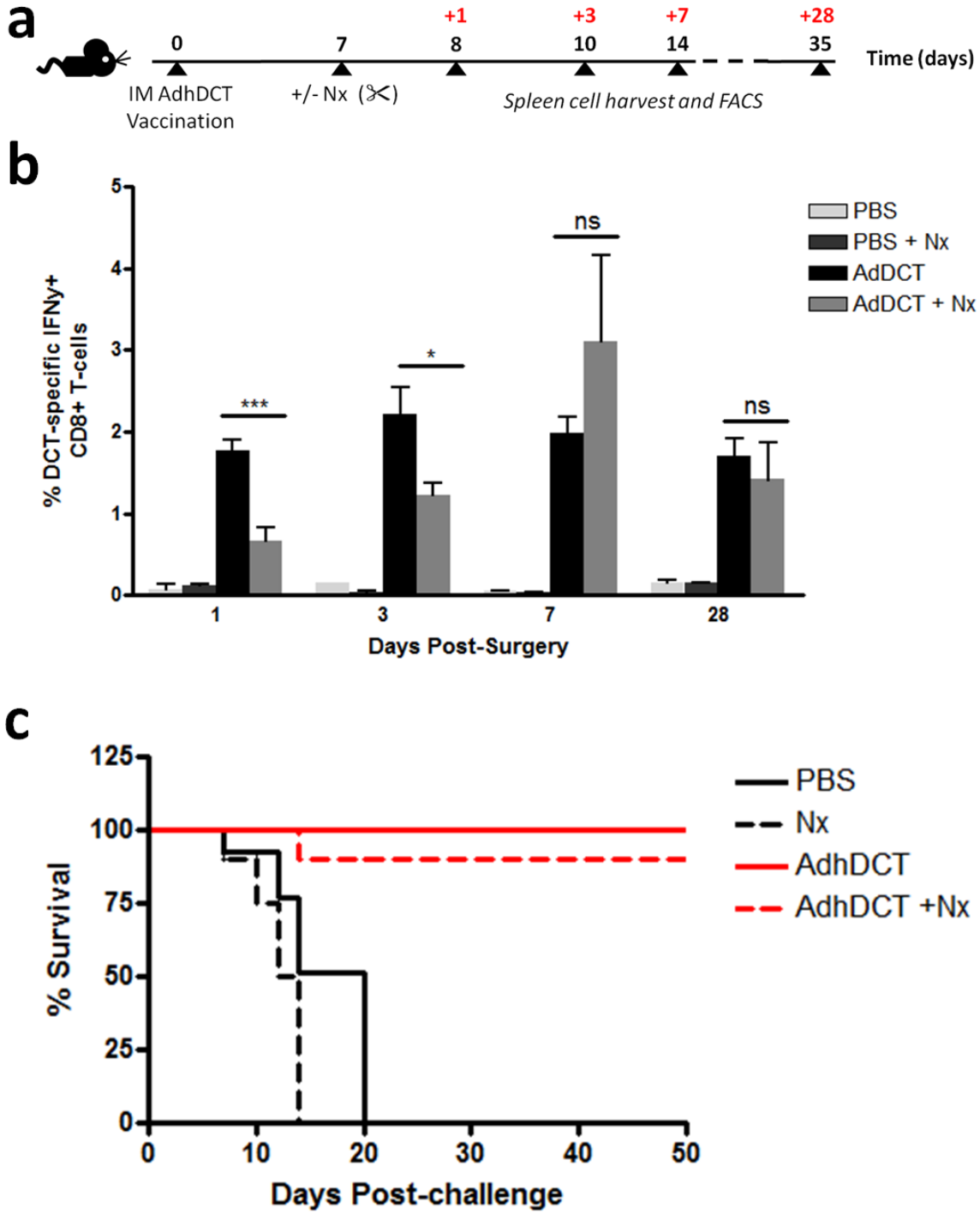

mouse 1
mouse 2
PBS
6
3
1
1
PBS + Nx
1
0
1
1
AdDCT
132
115
147
132
AdDCT + Nx
37
21
39
42

Figure 9. IFNγ ELISPOT of $DCT_{180-188}$ peptide-stimulated whole splenocytes 24 hours post-surgery. a) Experiment design. On day 0, C57/BL-6 mice (n=4/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 8, spleen cells were harvested and $4x10^5$ splenocytes were plated per ELISPOT well in duplicate. Splenocytes were restimulated *in vitro* with $DCT_{180-188}$ peptide for 24 hours and spot-forming units were counted. **b)** Number of IFNγ+ spots per $4x10^5$ splenocytes. **c)** Images of IFNγ ELISPOT wells from 2 representative mice in each treatment group. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis.*p<0.05, **p<0.01, ***p<0.001


a
0
7
+1
8
+3
10
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14
+28
35
Time (days)
IM AdhDCT Vaccination
+/- Nx (✂)
Spleen cell harvest and FACS
b
% DCT-specific IFNγ+ CD8+ T-cells
PBS
PBS + Nx
AdDCT
AdDCT + Nx
***
*
ns
ns
1
3
7
28
Days Post-Surgery
c
% Survival
PBS
Nx
AdhDCT
AdhDCT +Nx
Days Post-challenge

Figure 10. Frequency of $DCT_{180-188}$ peptide-stimulated CD8+ T-cells expressing IFNγ in the spleen 1, 3, 7, and 28 days post-surgery, and survival following SQ B16F10-LacZ challenge on day 28. a) Experiment timeline. On day 0, C57/BL-6 mice (n=4-5/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. Mice were endpointed on the indicated days for FACS analysis of spleens. A separate cohort of mice (n=7-8/group) were immunized IM with $1x10^6$ pfu AdhDCT or PBS. On day 7, these mice either underwent Nx or did not and were challenged 28 days post-surgery with SQ $3x10^5$ B16F10-LacZ in the right hind flank. Tumour outgrowth was monitored and mice were endpointed when tumour diameter reached 15mm. **b)** Proportion of $DCT_{180-188}$-specific CD8+ T-cells expressing IFNγ in the spleen at 1, 3, 7, and 28 days post-surgery. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis. **p<0.05, **p<0.01, ***p<0.001.* **c)** Kaplan-Meier survival curves of B16F10-LacZ tumour-bearing mice challenged 28 days post-surgery. Statistical significance was determined by log-rank tests.

immunized mice that underwent surgery in comparison to immunized mice that did not undergo surgery.

3.8 Surgical stress does not alter the frequency of T-cells, but does decrease T-cell numbers

Thus far, we have shown a significant attenuation of cytokine-expressing DCT-specific CD8+ T-cells. However, we also wanted to explore whether the significant disadvantage in tumour survival in surgically stressed immunized mice is due to a lack of a sufficient quantity of T-cells to mediate an anti-tumour effect as opposed to an intrinsic defect in T-cell activity. To test this hypothesis, we measured the frequency and number of DCT-specific CD8+ T-cells following surgery using a fluorescently labeled DCT peptide-loaded MHC-I tetramer. While in vitro peptide restimulation and FACS analysis of intracellular cytokines is a functional readout of the ability of DCT-specific T-cells to be

activated, tetramer staining directly quantifies T-cells with TCRs specific for a particular epitope.

In our model (**Figure 11a**), surgical stress did not affect the frequency of CD8+ T-cells in the spleen (**Figure 11b**), but did result in a 2-fold decrease in total CD8+ T-cell numbers (**Figure 11c**). Similarly, we also observed that the frequency of DCT-tetramer^{+} T-cells was not affected by surgical stress (**Figure 11d**). However, in contrast, surgical stress decreased the absolute number of DCT-specific CD8+ T-cells in the spleen, although not to the point of statistical significance (**Figure 11e**).

This may suggest that decreased post-operative tumour survival in immunized mice is not only due to the hyporesponsiveness of antigen-specific T-cells, but also partially due to the loss of T-cell numbers.

3.9 Surgical stress does not promote T-cell apoptosis, but does attenuate antigen-specific T-cell proliferation and T-cell infiltration into tumours.

In this regard, we explored possible mechanisms leading to decreased T-cell numbers. We measured whether surgical stress increased apoptosis of CD8+ T-cells by analyzing Annexin V and 7-AAD expression by flow cytometry. Annexin V is a phospholipid-binding protein which binds to phosphatidylserine (PS) to identify apoptotic cells. Upon initiation of apoptosis, PS translocates from the cytosolic side of the cell to the extracellular membrane, which is detectable with fluorescently labeled Annexin V. 7-AAD is a fluorescent viability dye with a strong affinity for DNA. In early stages of apoptosis, the plasma membrane excludes viability dyes, therefore cells which are Annexin V positive (and 7-AAD

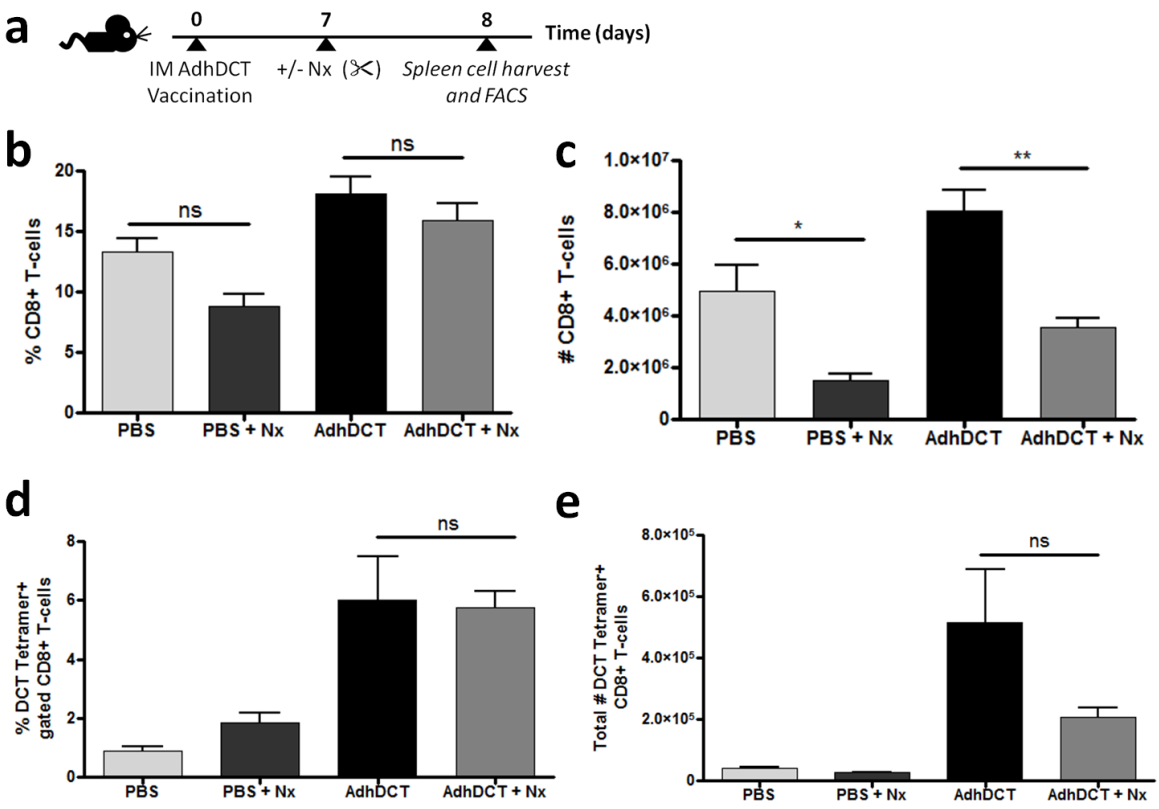

a
0
7
8
Time (days)
IM AdhDCT Vaccination
+/- Nx (✂)
Spleen cell harvest and FACS
b
% CD8+ T-cells
ns
ns
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
c
CD8+ T-cells
*
**
d
% DCT Tetramer+ gated CD8+ T-cells
e
Total # DCT Tetramer+ CD8+ T-cells

Figure 11. Frequency and absolute numbers of CD8+ T-cells and DCT-tetramer+ CD8+ T-cells in the spleen 24 hours post-surgery. a) Experiment design. On day 0, C57/BL-6 mice (n=5/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 8, spleen cells were isolated and fluorescently labelled and analyzed by FACS. **b)** Proportion of $CD8^+$ T-cells. **c)** Absolute number of $CD8^+$ T-cells. **d)** Proportion of $CD8^+$ T-cells that are $DCT_{180-188}$-$tetramer^+$ **e)** Absolute number of CD8+ T-cells that are $DCT_{180-188}$-$tetramer^+$. Statistical significance was determined by one-way ANOVA and Bonferroni post-hoc analysis. **p<0.05, **p<0.01, ***p<0.001*

negative) are in early stages of apoptosis. During late-stage apoptosis or necrosis, loss of cell membrane integrity allows Annexin V binding to cytosolic PS, as well as cell uptake of 7-AAD. While there is a significant decrease in the number of viable CD8+ T-cells, there is no corresponding increase in the number of CD8+ T-cells undergoing early or late apoptosis (**Figure 12a**).

Next, we looked at the possibility of a defect in T-cell proliferation by surgical stress. To this effect, we measured the incorporation of BrdU following DCT peptide restimulation in our immunization and surgery model (**Figure 12b**). We found that CD8+ T-cells from surgically stressed and immunized mice were significantly less proliferative in response to DCT peptide stimulation (**Figure 12c**). Additionally, since the viability assay demonstrated no increase in dead cells, we were interested in determining whether immune cell migration to the tumour was affected in surgically stressed mice (**Figure 12d**). To assess this, mice were vaccinated with AdhDCT or PBS, and one week later injected 3×10^5 B16lacZ cells mixed with a matrigel plug subcutaneously and performed surgery immediately. Matrigel was used in order to determine the immune cell profile within the tumour microenvironment. Matrigel plugs were harvested for flow cytometric analysis of infiltrating

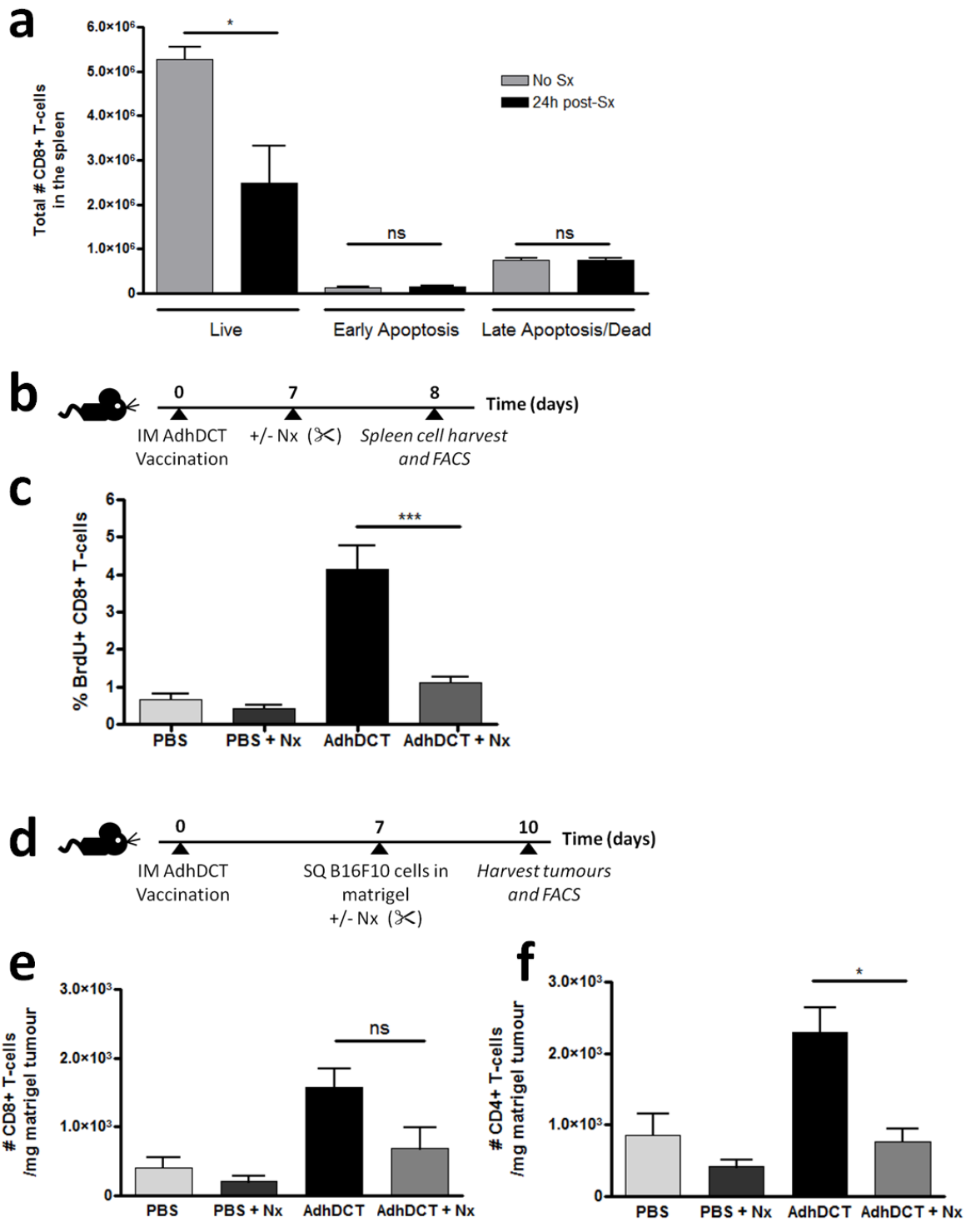

a
Total # CD8+ T-cells in the spleen
6.0×10⁶
5.0×10⁶
4.0×10⁶
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
*
ns
ns
No Sx
24h post-Sx
Live
Early Apoptosis
Late Apoptosis/Dead
b
0
7
8
Time (days)
IM AdhDCT Vaccination
+/- Nx (✂)
Spleen cell harvest and FACS
c
% BrdU+ CD8+ T-cells
6
5
4
3
2
1
0
***
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
d
0
7
10
Time (days)
IM AdhDCT Vaccination
SQ B16F10 cells in matrigel +/- Nx (✂)
Harvest tumours and FACS
e
CD8+ T-cells /mg matrigel tumour
3.0×10³
2.0×10³
1.0×10³
0
ns
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
f
CD4+ T-cells /mg matrigel tumour
3.0×10³
2.0×10³
1.0×10³
0
*
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

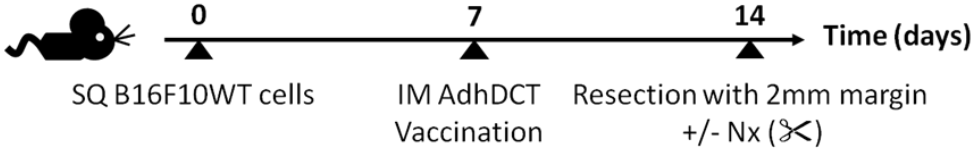
Figure 12. CD8+ T-cell apoptosis, proliferation, tumour infiltration following surgery. a) Absolute number of spleen CD8+ T-cells that are viable (7AAD- and Annexin V-), undergoing early apoptosis (7AAD- and Annexin V+), or late apoptosis (7AAD+ and Annexin V+) 24 hours post-surgery. **b)** Experiment outline. On day 0, C57/BL-6 mice (n=5/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, anesthetized mice either underwent full abdominal laparotomy and left nephrectomy (Nx) or did not undergo surgery. On day 8, isolated spleen cells were restimulated in vitro with DCT180-188 peptide and labelled with BrdU. Splenocytes were stained with antibodies and assessed by flow cytometry. **c)** Frequency of CD8+ T-cells incorporating BrdU. **d)** Experiment outline. On day 0, C57/BL-6 mice (n=3/group) were immunized IM with $1x10^7$ pfu AdhDCT or PBS. On day 7, mice were challenged with subcutaneous B16-F10.LacZ tumours mixed with matrigel prior to surgery. On day 10, matrigel plugs were removed and assessed for immune cell infiltration by flow cytometry. **e)** Number of CD8a+ CD3+ T-cells per mg of tumour. **f)** Number of CD4+CD3+ T-cells per mg of tumour. *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA and Student's t-test.

lymphocytes 3 days post-surgery. Our data demonstrates that surgically stressed mice have decreased CD8+ T-cell (**Figure12e**) and CD4+ T-cell (**Figure 12f**) populations in the tumour, indicating that surgery may affect the migration of these immune cell subsets to the tumour site and potentially prevent flank tumour clearance.

3.10 Surgical stress attenuates neoadjuvant AdhDCT vaccination and potentiates tumour growth in a clinically relevant model of minimal residual disease

So far, we have demonstrated that surgical stress attenuates antigen-specific T-cell immunity in the prophylactic immunization model and potentiates tumour growth. However, we wanted to assess the effect of surgery in a clinically-relevant therapeutic model of minimal residual disease. B16F10 tumours were injected into the flank and allowed to grow for 1 week until neoadjuvant vaccination with AdhDCT on day 7. On day 14, all mice underwent partial tumour resection leaving behind a 2mm positive margin.

a


0
7
14
Time (days)
SQ B16F10WT cells
IM AdhDCT Vaccination
Resection with 2mm margin +/- Nx (✂)

b

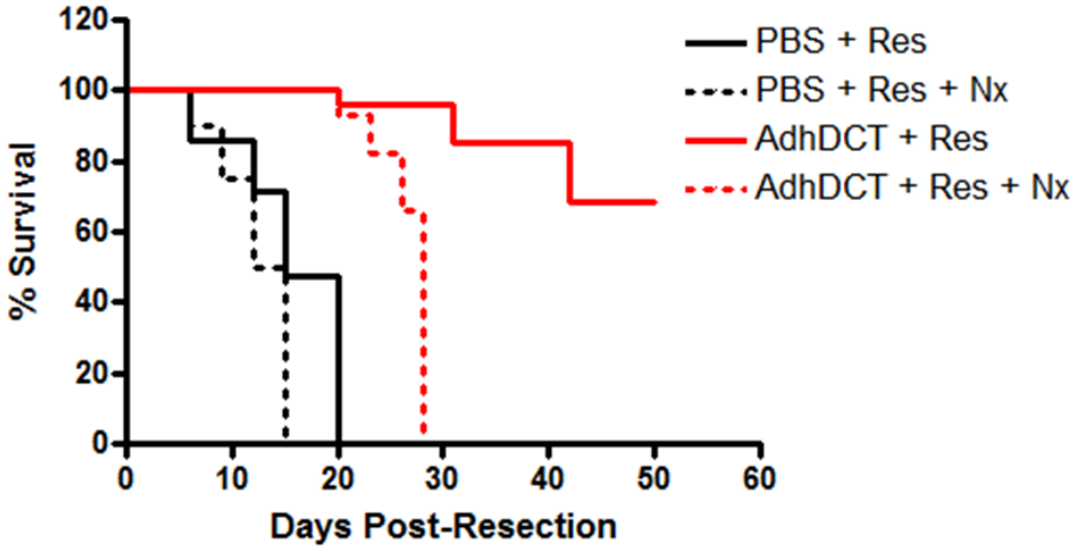

% Survival
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Days Post-Resection
PBS + Res
PBS + Res + Nx
AdhDCT + Res
AdhDCT + Res + Nx

Figure 13. Surgical stress abrogates neoadjuvant AdhDCT vaccination in a B16F10 model of minimal residual disease resulting in post-operative tumour recurrence. a) Experimental timeline. On day 0, C57BL/6 mice (n=7-8/group) were injected with $1x10^5$ SQ B16F10 tumours in the right hind flank. On day 7, mice were immunized with $1x10^7$ pfu AdhDCT. On day 14, tumors were resected (Res) with a 2mm margin with or without nephrectomy (Res+Nx). Tumour outgrowth was monitored over time and mice were endpointed when tumour diameter reached 15mm. **b)** Kaplan-Meier survival curves of B16F10 tumour-bearing mice. Statistical significance was determined by log-rank tests. **p<0.05, **p<0.01, ***p<0.001.*

Similar to the studies above, we also incorporated into this model the effects of a major surgical procedure by subjecting the mice to a laparotomy and nephrectomy (**Figure 13a**). In this model, 75-100% of AdhDCT-immunized mice that underwent partial resection alone were protected from tumour outgrowth. In contrast, the effects of the major surgical stress were dramatic with 100% of mice developing a tumour at a rate similar to those mice that received PBS instead of Ad-hDCT vaccination (**Figure 13b**).

While the postoperative period provides a window of opportunity for cancer cells to metastasize and grow, it also provides a window of opportunity to intervene, by supporting or further stimulating the immune system to reverse the immunosuppressive effects of surgery and, in doing so, attenuate the development of cancer recurrences[85,86]. Clinical trials of preoperative non-specific immune stimulation with low dose IFNα[87] or IL-2[88,89] have demonstrated less NK and T-cell suppression following surgery and modest improval of survival.

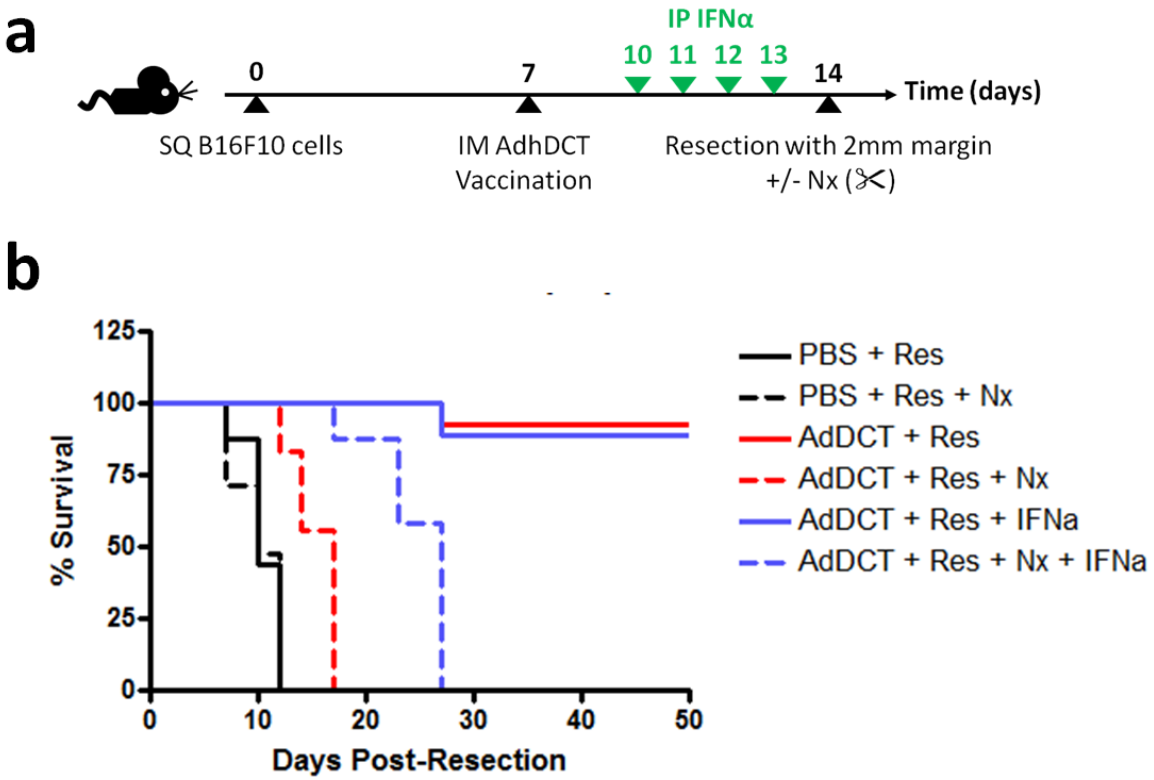

a
IP IFNα
10 11 12 13
0
7
14
Time (days)
SQ B16F10 cells
IM AdhDCT
Vaccination
Resection with 2mm margin
+/- Nx (✂)
b
% Survival
125
100
75
50
25
0
0
10
20
30
40
50
Days Post-Resection
PBS + Res
PBS + Res + Nx
AdDCT + Res
AdDCT + Res + Nx
AdDCT + Res + IFNa
AdDCT + Res + Nx + IFNa

Figure 14. Preoperative treatment with multi-dose IFNa can rescue the effects of surgical stress on tumour growth in a B16F10 model of minimal residual disease. a) Experimental timeline. On day 0, C57BL/6 mice (n=7-8/group) were injected with $1x10^5$ SQ B16F10 tumours in the right hind flank. On day 7, mice were immunized with $1x10^7$ pfu AdhDCT. On days 10, 11, 12, and 13, mice were given 1 high dose (10,000 IU/mouse) and 3 low doses (2000 IU/mouse) of murine IFN-α IP. On day 14, tumors were resected (Res) with a 2mm margin with or without nephrectomy (Res+Nx). Tumour outgrowth was monitored over time and mice were endpointed when tumour diameter reached 15mm. **b)** Kaplan-Meier survival curves of B16F10 tumour-bearing mice. Statistical significance was determined by log-rank tests. **p<0.05, **p<0.01, ***p<0.001.*

Since IFNα is less toxic than IL-2 in patients, we chose to test pre-operative IFNα therapy in our positive margin model (**Figure 14a**). We also chose IFNα because type I interferons are known to be induced following oncolytic virus injection[16]. Therefore, any improvement in survival conferred by IFNα treatment provides the rationale to use a heterologous oncolytic virus vaccine boost in the perioperative setting. Our survival study illustrates that preoperative IFNα can partially restore the potency of a therapeutic vaccination in the surgically stressed mice, though eventually 100% of mice succumb to tumour outgrowth (**Figure 14b**).

However, it is clear that the immunosuppressive effects of surgery can be modulated with preoperative immunotherapies that interfere with the mechanisms responsible for cancer growth and metastases following surgery and may, in fact, restore the potency of a cancer vaccine.

4 - DISCUSSION

Surgical resection is the mainstay of therapy for most solid malignancies but despite complete resection, many patients harbour MRD and ultimately die of a recurrence[90]. Cancer vaccines are best suited to eradicate MRD[72,91,92], providing a strong rationale to combine surgery and immunotherapy. In the present study, we have demonstrated that the profound suppression of tumour antigen-specific T-cell immunity following major surgery potentiates the development of cancer metastases and local recurrence in a pre-immunized mouse model of melanoma.

Previous studies[80-82,93] have shown that immunization with AdhDCT, a melanoma-specific adenoviral gene therapy vector, generates a robust DCT-specific T-cell immune response and can significantly protect mice from intercranial, intravenous, and subcutaneous melanoma tumours.

Interestingly, intravenously-injected melanoma cells are more susceptible to NK cell-mediated death in comparison to subcutaneous melanoma, in large part due to differences in tumour microenvironment and immune cell proportions[94,95]. Priming of naive T-cells is mainly mediated by DCs, but recently, research has shown that NK cells are recruited to lymph nodes following immune stimulation and help prime T-cells[96]. This may be why AdhDCT has no effect on tumour burden in the NK-depletion and intravenous tumour challenge model, since T-cells may not be adequately primed.

Therefore, we proceeded to assess the effects of surgical stress in AdhDCT-immunized mice challenged with a flank tumour. Remarkably, the effects of surgical stress

were dramatic with 100% of mice developing a tumour recurrence at a rate similar to those mice that received PBS instead of Ad-hDCT vaccination. Surgery-induced dysfunction of T-cells was further corroborated with tumour survival data in athymic CD-1 nude mice which demonstrated no significant survival differences between surgically-stressed and non-surgically-stressed AdhDCT-immunized mice, despite having intact NK cells. Previous studies by our group and others have demonstrated that the suppression of NK cell activity after surgery has been shown to promote tumour development[41-44]. However, we observed in our CD-1 nude mice that despite having intact NK cells, there was no difference in tumour survival between surgically-stressed and non-surgically-stressed mice, suggesting that in a flank challenge model, T-cells play a more essential role in mediating anti-tumour immunity.

Moreover, adoptive transfer of global CD3+ T-cell populations from surgically-stressed mice was unable to protect recipient mice from a subcutaneous melanoma challenge, further substantiating our hypothesis that surgical stress results in an inherent defect of effector antigen-specific CD8+ T-cells. Importantly, we also observed the attenuation of anti-tumour immunity by surgical stress in a CT26 colorectal cancer model, suggesting that surgery-induced dysfunction of tumour-specific immunity is not exclusive to our melanoma model.

We subsequently characterized the effect of major surgery on DCT-specific T-cell immunity by intracellular flow cytometry and demonstrated that surgical stress resulted in a significant decrease in IFNγ-, TNFα-, and GranzymeB-secreting DCT-specific $CD8^{+}$T-cells beginning 24 hours after surgery and lasting approximately 7-10 days. A similar effect on

DCT-specific immunity was demonstrated using an ELISpot assay for IFNγ secretion on isolated splenic $CD8^+$T-cells.

Interestingly, even when splenocytes were non-specifically stimulated with phorbol myristate acetate (PMA) and ionomycin, both mitogens that activate the NFAT (nucelar factor of activated T-cells) transcription factor in T lymphocytes[84], secretion of IFNγ was significantly attenuated in AdhDCT-immunized and surgically stressed mice. This, along with our tetramer analysis, may suggest that surgery-induced dysfunction of T-cells is independent of the TCR (T-cell receptor) and could be due to other external factors. Generalized T-cell dysfunction has been documented in patients following major surgery, including a significant decrease in $CD4^+$ and $CD8^+$ T-cell numbers[97,98] associated with increased apoptosis[99-101], and a severe defect in cytokine secretion (IFNγ and TNFα)[97,102] and proliferation following *in-vitro* stimulation[102]. The mechanisms responsible for these effects are likely multifactorial and have been explored in both preclinical and clinical studies (reviewed in *Shakhar et al*[103]).

Cancer patients generate a baseline population of tumour-specific CD8+ T-cells. However, as tumours progress in their development, the cytolytic activity of these tumour-specific CD8+ T-cells is impaired due to tumour-induced antigenic tolerance and increased infiltration of immunosuppressive tumour-specific CD4+ Treg cells at the tumour site[6]. Accumulating clinical data show that the presence of tumour-infiltrating lymphocytes is a strong prognostic factor associated with both freedom from disease and overall survival[104-108]. To this effect, we assessed tumour-infiltrating lymphocyte populations following surgery. We observed that immunization with AdhDCT increases the infiltration of CD8+ T-

cells in the tumour. Notably, we found a decrease in CD4+ T-cells and CD8+ T-cells, suggesting that surgical stress prevents the migration of these effector cells to the tumour site. Tregs play a pivotal role in immunological suppression[109]. Studies have shown that depletion of Tregs by CD25 or CD4 depletions prior to development of immune responses results in enhanced immunological responses against tumors[110]. Recently, it has been shown that an increased presence of regulatory T-cells in the peripheral blood of surgical patients correlates with the degree of surgical stress[111]. Though we did not observe an expansion of Tregs in our mouse surgical stress model (Lee Hwa-Tai unpublished data), we have observed a marked postoperative expansion of MDSCs, an alternate suppressive immune cell population, as well as postoperative upregulation of TGFβ. MDSCs represent a heterogeneous population of immature myeloid cells that suppress T cell functions through both antigen-dependent and independent means. They deplete arginine and secrete reactive oxygen species, to which effector T cells have a heightened sensitivity that leads to their anergy or apoptosis[112]. MDSCs also produce TGFβ and IL-10, reflecting the critical role of these cytokines.

The immediate postoperative period provides fertile ground for cancer growth and metastases. Despite this, it remains a therapeutic window of opportunity that is largely ignored in our current cancer treatment paradigm. Cancer treatments aimed at targeting the immunosuppressive mechanisms responsible for the prometastatic effects of surgery, such as cancer vaccination and perioperative immunomodulation, can reduce recurrences and improve survival in surgical cancer patients. This therapeutic strategy has the potential

to impact over 65,000 Canadians who undergo surgical resection of their solid tumour every year[1], however requires further study and optimization.

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111. Saito Y, Shimada M, Utsunomiya T, et al. Regulatory T cells in the blood: A new marker of surgical stress. *Surg Today*. 2013;43(6):608-612.

112. Poschke I, Mougiakakos D, Kiessling R. Camouflage and sabotage: Tumor escape from the immune system. *Cancer Immunol Immunother*. 2011;60(8):1161-1171.

CONTRIBUTIONS OF COLLABORATORS

The following colleagues are recognized as contributing to the work detailed in this thesis. Christiano Tanese de Souza performed IV and SQ injections for all *in vivo* mouse experiments. Casey Lansdell provided technical assistance for the tetramer (Figure 11), T-cell apoptosis (Figure 12a), and T-cell BrdU proliferation (Figure 12b,c) assays. Dr. Brian Lichty and Dr. Jonathan Bramson from McMaster University were close collaborators on the entire project and kindly provided AdhDCT virus.

Abhirami Anu Ananth
CURRICULUM VITAE

ACADEMIC BACKGROUND

2011-2014 **Masters of Science (Biochemistry)**, University of Ottawa

2007-2011 **Bachelor of Science** with Honours in Biopharmaceutical Sciences, University of Ottawa

AWARDS, HONOURS

2013 **GSAED Academic Project Fund**, University of Ottawa
Awarded for the best graduate student abstract submitted for publication at a conference ($300)

2013 **Student Conference Travel Grant**, Faculty of Graduate and Postdoctoral Studies, University of Ottawa
Awarded to graduate students for excellent research potential ($120)

2013 **Department of Biochemistry, Microbiology and Immunology Student Travel Award**, University of Ottawa
Awarded to exceptional graduate students who display commitment to research and academic excellence ($550)

2013 **Travel Award**, Seventh International Meeting On Replicating Oncolytic Virus Therapeutics
Awarded to the most outstanding abstracts presented by conference attendees ($300)

2013 **Biochemistry Program Travel Award**, University of Ottawa
Awarded to graduate students in the Biochemistry program who display excellent academic merit and research potential ($550)

2012 **Masters Student Poster Competition (2nd Place)**, Ottawa Hospital Research Institute Research Days
Awarded to graduate students who demonstrate outstanding research capabilities and exceptional aptitude for scientific discourse ($250)

2011-2013 **Graduate Studies Scholarship**, University of Ottawa
Research funding awarded to high-calibre graduate students ($30,000)

2010 **NSERC Industrial Research Award**, NSERC
Awarded to undergraduate students to participate in collaborative research opportunities in partnership with universities and pharmaceutical companies ($4500)

2008 **Boehringer Ingelheim Undergraduate Research Award**, University of Ottawa
Awarded to second-year undergraduate students who demonstrate exceptional research potential during their first year of university

2007-2011 **Dean's Honour List**, University of Ottawa
Awarded to undergraduate students who demonstrate academic excellence

2007-2011 **Renewable Admission Scholarship**, Faculty of Science, University of Ottawa
Awarded to matriculating high school students who exhibit and maintain a stellar academic record ($16,000)

2007-2008 **Undergraduate Research Scholarship**, University of Ottawa
Awarded to first-year undergraduate students based on academic performance and research potential in basic sciences ($10,000)

2003-2007 **OCDSB Silver Medal Award**, Ottawa-Carleton District School Board, Ottawa, Ontario
Awarded to high school students who display exceptional academic merit and place in the top 10% of high school students city-wide in overall grades

2003-2007 **Academic Achievement Award**, Lisgar Collegiate Institute, Ottawa, Ontario
Awarded to students who demonstrate exceptional academic merit in high school

JOURNAL PUBLICATIONS

Published:

1. Vähä-Koskela MJ, Le Boeuf F, Lemay C, De Silva N, Diallo JS, Cox J, Becker M, Choi Y, **Ananth A**, Sellers C, Breton S, Roy D, Falls T, Brun J, Hemminki A, Hinkkanen A, Bell JC. 2013. *Resistance to two heterologous neurotropic oncolytic viruses, Semliki Forest virus and vaccinia virus, in experimental glioma.* Journal of Virology 87(4): 2363-2366.

2. Tai LH, de Souza CT, Bélanger S, Ly L, Alkayyal AA, Zhang J, Rintoul JL, **Ananth AA**, Lam T, Breitbach CJ, Falls TJ, Kirn DH, Bell JC, Makrigiannis AP, Auer RA. 2013. *Preventing*

postoperative metastatic disease by inhibiting surgery-induced dysfunction in natural killer cells. Cancer Research 73(1): 97-107.

3. Tai LH, Zhang J, Scott KJ, Tanese de Souza C, Alkayyal AA, **Ananth AA**, Sahi S, Adair RA, Bakur Mahmoud A, Sad S, Bell JC, Makrigiannis AP, Melcher AA, Auer R. 2013. *Perioperative influenza vaccination reduces post-operative metastatic disease by reversing surgery-induced dysfunction in natural killer cells.* Clinical Cancer Research [Epub ahead of print].

4. Tai LH, Tanese de Souza C, Sahi S, Zhang J, Alkayyal AA, **Ananth AA**, Auer RC. 2013. *A mouse tumor model of surgical stress to explore the mechanisms of postoperative immunosuppression and evaluate novel perioperative immunotherapies.* Journal of Visualized Experiments [In Press].

Submitted:

1. Zhang J, Tai L-H, Ilkow C, de Souza CT, Alkayyal AA, **Ananth AA**, Lefebvre C, Stojdl D, Bell JC, Auer RA. *Rhabdovirotherapy efficacy with natural killer cell stimulating non-replicating oncolytic Maraba MGI.* Revision requested May 13 2013. Molecular Therapy. MT-T-13-254.

In Preparation:

1. **Ananth AA,** Seth R, Tai LH, Alkayyal AA, Zhang J, Tanese de Souza C, Bell JC, Auer RA. Perioperative metastases are augmented by sepsis but not hypovolemia or hypothermia in a murine model of colorectal cancer. In preparation for submission to Annals of Surgery.

2. **Ananth AA,** Tai LH, Parato K, Tanese de Souza C, Alkayal AA, Zhang J, Pol J, Bridle B, Stojdl DF, Atkins HL, Bell JC, Lichty BD, Auer RA. Surgical stress attenuates pre-existing anti-tumour immunity resulting in postoperative metastases and local recurrence in a murine model. In preparation for submission to Cancer Immunology Research.

ABSTRACTS PRESENTED

1. **Ananth AA**, Tai LH, Tanese de Souza C, Alkayyal AA, Zhang J, Parato K, Pol J, Bridle B, Stojdl DF, Atkins HL, Lichty BD, Bell JC, Auer RA. Surgical stress attenuates pre-existing anti-tumour immunity resulting in postoperative metastases and local recurrence in a murine model. Poster presentation to the Canadian Cancer Research Conference, Toronto, Ontario, November 2013.

2. **Ananth AA**, Tai LH, Parato K, Tanese de Souza C, Pol J, Bridle B, Stojdl DF, Atkins HL, Bell JC, Lichty BD, Auer RA. Surgical stress attenuates pre-existing anti-tumour immunity resulting in postoperative metastases and local recurrence in a murine model. Oral Presentation for the CAGS Canadian Surgery Forum, Ottawa, Ontario, September 2013.

3. **Ananth AA**, Tai LH, Tanese de Souza C, Alkayyal AA, Zhang J, Parato K, Pol J, Bridle B, Stojdl DF, Atkins HL, Lichty BD, Bell JC, Auer RA. Surgery-induced vaccine dysfunction in a murine model of melanoma can be rescued with perioperative oncolytic rhabdovirus. Submitted to the Seventh International Meeting for Oncolytic Virus Therapeutics, Quebec City, Quebec, June 2013.

4. **Ananth AA**, Tai LH, Tanese de Souza C, Alkayyal AA, Zhang J, Parato K, Pol J, Bridle B, Stojdl DF, Atkins HL, Lichty BD, Bell JC, Auer RC. Surgical stress attenuates pre-existing anti-tumour immunity resulting in postoperative metastases and local recurrence in a murine model. Podium Presentation at the Division of General Surgery Research Day, University of Ottawa, Ottawa, Ontario, April 2013.

5. **Ananth AA**, Tai LH, Tanese de Souza C, Bridle B, Atkins HL, Lichty BD, Bell JC, Auer RC. Surgery-induced Vaccine Dysfunction in a Murine Model of Melanoma. Oral Presentation at the Biochemistry, Microbiology & Immunology Seminar Day, University of Ottawa, Ottawa, Ontario, March 2013.

6. **Ananth AA**, Tai LH, Tanese de Souza C, Bridle B, Atkins HL, Lichty BD, Bell JC, Auer RC. Surgery-induced Vaccine Dysfunction in a Murine Model of Melanoma. Poster Presentation at the Ottawa Hospital Research Institute Symposium, St. Elias Centre, Ottawa, Ontario, November 2012.

7. **Ananth AA**, Tai LH, Tanese de Souza C, Bridle B, Atkins HL, Lichty BD, Bell JC, Auer RC. Surgery-induced Vaccine Suppression in Murine Melanoma. Poster Presentation at the Biochemistry, Microbiology & Immunology Research Symposium, University of Ottawa, Ottawa, Ontario, May 2012.

8. **Ananth AA**, Tai LH, Tanese de Souza C, Bridle B, Atkins HL, Lichty BD, Bell JC, Auer RC. Impairment of Vaccine-Induced CD8+ T Cells by Surgery. Poster Presentation at the Division of General Surgery Research Day, University of Ottawa, Ottawa, Ontario, April 2012.

9. **Ananth AA**, Seth R, Tai LH, Tanese de Souza C, Atkins HL, Bell JC, Auer RC. Perioperative Sepsis Enhances the Development of Metastases. Poster Presentation at the Ottawa Hospital Research Institute Symposium, St. Elias Centre, Ottawa, Ontario, November 2011.

10. **Ananth AA**, Vaha-Koskela M, LeBoeuf F, Bell JC. The development of novel recombinant oncolytic Vaccinia viruses for combined virotherapy and immunotherapy of B16 melanoma cancer. Poster Presentation at Biochemistry, Microbiology & Immunology Undergraduate Poster Symposium, University of Ottawa, Ottawa, Ontario, April 2011.

11. **Ananth AA**, Vaha-Koskela M, Bell JC. Induction of a Th17 immune response with cytokine-expressing oncolytic Vaccinia virus. Oral Presentation for the Ottawa Hospital Research Institute Summer Research Program, Sprott Centre for Stem Cell Research, Ottawa, Ontario, August 2009.

RESEARCH PROJECTS

2012	**UV-Inactivated Oncolytic Maraba Rhabdovirus is a Potent Stimulator of Innate Immunity in a Perioperative Model of Cancer Metastases** Dr. Lee Hwa Tai and Dr. Rebecca Auer, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario
2011	**Perioperative Flu Vaccine Adminstration Treatment Rescue** Dr. Lee Hwa Tai and Dr. Rebecca Auer, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario
2011-2013	**Assessment of Natural Killer Cell Dysfunction in a Murine Model of Surgical Stress** Dr. Lee Hwa Tai and Dr. Rebecca Auer, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario
2011-2013	**Identification of Candidate Tumor Peptides in B16-Subtype Melanoma for the Development of Target-specific Oncolytic Viruses** Dr. Harry Atkins, Dr. John Bell, and Dr. Rebecca Auer, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario
2011-2013	**Assessment of Surgery-Induced Vaccine Dysfunction in a Murine Model of Melanoma** Dr. Rebecca Auer and Dr. John Bell, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario
2011-2012	**Evaluation of Perioperative Risk Factors Contributing to Pulmonary Metastases in a Murine Model** Dr. Rebecca Auer and Dr. John Bell, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario
2010	**Development of Recombinant Oncolytic *Vaccinia* Viruses to Target B16 Melanoma** Dr. John Bell, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario
2009	**Maintenance of Good Manufacturing Practices for Production of Clinical-Grade Oncolytic Virus Therapeutics** Dr. John Bell, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario

2009 **Assessment of Glioblastoma Multiforme Treatment Efficacy by Oncolytic Viruses in a Murine Model**
Dr. John Bell, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario

2008 **Assessment of Genetic Polymorphisms in Sulfate-Reducing Bacteria Recovered From European Mines**
Dr. Danielle Fortin, Department of Earth Sciences, University of Ottawa, Ottawa, Ontario

2007 **Molecular Genotyping of Sulfate-Reducing Bacteria in European Mine Tailings**
Dr. Jutta Meier and Dr. Danielle Fortin, Department of Earth Sciences, University of Ottawa, Ottawa, Ontario

LEADERSHIP AND COMMUNITY ACTIVITIES

1997-present **Classical South Indian Music Singer**, Ottawa, Ontario
Regularly performed at Hindu Temple of Ottawa-Carleton and at numerous venues around the city

2011-2014 **Event Organizer**, Graduate Student Association, University of Ottawa, Ottawa, Ontario
Planned and organized educational and social activities for graduate students in the Department of Biochemistry, Microbiology, and Immunology

2010-2014 **Attendant**, Cancer Therapeutics Journal Club, Ottawa Hospital Research Institute, University of Ottawa, Ottawa, Ontario
Attended and discussed journal articles published in peer-reviewed journals as part of the Graduate Medical Education program

2009-2014 **Volunteer**, Royal Ottawa Hospital, Ottawa, Ontario
Assisted and provided care to patients in the inpatient geriatric ward of the hospital under supervision by nurses

2012-2013 **Vice-President**, Department of Biochemistry, Microbiology, and Immunology Graduate Student Association, University of Ottawa
Responsible for organizing and implementing student-led initatives to improve the educational experiences of graduate students within the department

2011-2013 **Writer**, BMI Bulletin, University of Ottawa

	Regular contributing author for monthly student-run newsletter for the department of Biochemistry, Microbiology and Immunology, focusing on student issues
2013	**Volunteer**, Terry Fox Research Institute 4th Annual Scientific Meeting, Delta Ottawa City Centre, Ottawa *Responsible for welcoming conference participants and monitoring plenary sessions*
2012	**Group Leader**, Relay for Life Lab Team *Organized fundraising efforts within the laboratory group in support of the Cancer Relay for Life charity event*
2010	**International Volunteer**, Amar Seva Sangam Rehabilitation Centre for Disabled Children, Tamil Nadu, India *Worked with children with mental and physical disabilities during speech therapy sessions and organized recreational activities*
2009-2011	**Volunteer**, Civic Hospital, Ottawa, Ontario *Assisted patients and family members in the Emergency Department of the hospital under supervision by nurses*
2008	**Volunteer**, Canadian National Institute for the Blind, Ottawa, Ontario *Advocated for assistance programs for the visually-impaired in my neighborhood*
2008-2009	**Herbarium Assistant**, University of Ottawa, Ottawa, Ontario *Tended and cultivated endangered plant species from Africa, Bosnia, and South America for use in health research and complementary medicine*
2005-2008	**English and Math tutor**, Kumon Education Centre, Ottawa *Volunteered as a tutor in English and Math for underprivileged children in a south Ottawa neighborhood*
2003-2008	**Viola Performer**, Ottawa *Played western classical music at venues across Ottawa for social and community functions*
2001-2005	**Piano Performer**, Ottawa *Played western classical music at musical competitions across Ottawa*